UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 10,106,603 B2
APPLICATION NO. : 15/988463
DATED : October 23, 2018
INVENTOR(S) : Stuart Alexander Cook et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims

At Column 93, Claim 1, Lines 15-16, the text:
"A method of treating fibrosis in the human subject, the method comprising administering to a human subject in"
Should be replaced with the text:
--A method of treating fibrosis in a human subject, the method comprising administering to the human subject in--.

Signed and Sealed this
Fifth Day of February, 2019

Andrei Iancu
Director of the United States Patent and Trademark Office

Docket No.: M0546.70012US01
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Stuart Alexander Cook
Application No.:	15/988,463
Confirmation No.:	7597
Filed:	May 24, 2018
Patent No.:	10,106,603
Issued:	October 23, 2018
For:	TREATMENT OF FIBROSIS
Examiner:	P. M. Mertz
Art Unit:	1646

Certificate of Electronic Filing under 37 C.F.R. § 1.8
I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: January 10, 2019 Electronic Signature for Nancy J. Arsenault: /Nancy J. Arsenault/

REQUEST FOR CERTIFICATE OF CORRECTION PURSUANT TO 37 CFR 1.322

Attention: Certificate of Correction Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Upon reviewing the above-identified patent, Patentee noted an error which should be corrected.

In the Claims:

At column 93, Claim 1, lines 15-16, the text:

"A method of treating fibrosis in the human subject, the method comprising administering to a human subject in" should be replaced with the text --A method of treating fibrosis in a human subject, the method comprising administering to the human subject in--.

This error was made by the USPTO as evidenced by the claims in the Preliminary Amendment filed by Applicant on May 24, 2018 and the Examiner's Amendment to the claims in the Notice of Allowance dated August 3, 2018. See, in particular, claim 1 on page 3 of the Preliminary Amendment and the Examiner's Amendment to claim 1 on page 2 of the Notice of Allowance. Accordingly, no fee is required.

Transmitted herewith is a proposed Certificate of Correction effecting such amendment. Patentee respectfully solicits the granting of the requested Certificate of Correction.

Applicant believes no fee is due with this request. However, if a fee is due, please charge Deposit Account No. 23/2825 under Docket No. M0546.70012US01 from which the undersigned is authorized to draw.

Dated: January 10, 2019

Respectfully submitted,

By /Amy McMahon Krom/
Amy McMahon Krom, PhD
Registration No.: 73,073
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

PTO/SB/44 (09-07)
Approved for use through 01/31/2020. OMB 0651-0033
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

(Also Form PTO-1050)

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 10,106,603

APPLICATION NO. : 15/988,463

ISSUE DATE : October 23, 2018

INVENTOR(S) : Stuart Alexander Cook et al.

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims:

At column 93, Claim 1, lines 15-16, the text:

"A method of treating fibrosis in the human subject, the method comprising administering to a human subject in" should be replaced with the text --A method of treating fibrosis in a human subject, the method comprising administering to the human subject in--.

MAILING ADDRESS OF SENDER (Please do not use Customer Number below):
Amy McMahon Krom, PhD
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206

Electronic Acknowledgement Receipt

EFS ID:	34817382
Application Number:	15988463
International Application Number:	
Confirmation Number:	7597
Title of Invention:	TREATMENT OF FIBROSIS
First Named Inventor/Applicant Name:	Stuart Alexander Cook
Customer Number:	23628
Filer:	Amy McMahon Krom/Nancy Arsenault
Filer Authorized By:	Amy McMahon Krom
Attorney Docket Number:	M0546.70012US01
Receipt Date:	10-JAN-2019
Filing Date:	24-MAY-2018
Time Stamp:	13:20:06
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	M054670012US01-TRN-AM.pdf	26388 3c2b489fb1de1d2bb89a766ab2782b5a7d2739da	no	1

Warnings:

Information:					
2	Request for Certificate of Correction	M054670012US01-RFCOC-AM.pdf	28583 175508ef0300724ffec8aef7ba18c1b068430f00	no	2
Warnings:					
Information:					
3	Request for Certificate of Correction	M054670012US01-COC-AM.pdf	17936 105b2c1659128d7a495196d2a0d429eae687c991	no	1
Warnings:					
Information:					
			Total Files Size (in bytes):	72907	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM *(to be used for all correspondence after initial filing)*			
		Application Number	Patent#: 10,106,603
		Filing Date	Issued: October 23, 2018
		First Named Inventor	Stuart Alexander Cook
		Art Unit	1646
		Examiner Name	P. M. Mertz
Total Number of Pages in This Submission		Attorney Docket Number	M0546.70012US01

ENCLOSURES (***Check all that apply***)

☐ Fee Transmittal Form	☐ Drawing(s)	☐ After Allowance Communication to TC
☐ Fee Attached	☐ Licensing-related Papers	☐ Appeal Communication to Board of Appeals and Interferences
☐ Amendment/Reply	☐ Petition	☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
☐ After Final	☐ Petition to Convert to a Provisional Application	☐ Proprietary Information
☐ Affidavits/declaration(s)	☐ Power of Attorney, Revocation Change of Correspondence Address	☐ Status Letter
☐ Extension of Time Request	☐ Terminal Disclaimer	☒ Other Enclosure(s) (please Identify below):
☐ Express Abandonment Request	☐ Request for Refund	Request for Certificate of Correction
☐ Information Disclosure Statement	☐ CD, Number of CD(s) _____	Certificate of Correction
☐ Certified Copy of Priority Document(s)	☐ Landscape Table on CD	
☐ Reply to Missing Parts/ Incomplete Application	Remarks	
☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53		

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Amy McMahon Krom/		
Printed name	Amy McMahon Krom, PhD		
Date	January 10, 2019	Reg. No.	73,073

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: January 10, 2019 Electronic Signature for Nancy J. Arsenault: /Nancy J. Arsenault/

6756096.1

PTO/SB/47 (03-09)
Approved for use through 07/31/2018. OMB 0651-0016
U.S. Patent and Trademark Office; U. S. DEPARTMENT OF COMMERCE

"FEE ADDRESS" INDICATION FORM

Address to:
Mail Stop M Correspondence
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

- OR -

Fax to:
571-273-6500

INSTRUCTIONS: The issue fee must have been paid for application(s) listed on this form. In addition, only an address represented by a Customer Number can be established as the fee address for maintenance fee purposes (hereafter, fee address). A fee address should be established when correspondence related to maintenance fees should be mailed to a different address than the correspondence address for the application. **When to check the first box below**: If you have a Customer Number to represent the fee address. **When to check the second box below**: If you have no Customer Number representing the desired fee address, in which case a completed Request for Customer Number (PTO/SB/125) must be attached to this form. For more information on Customer Numbers, see the Manual of Patent Examining Procedure (MPEP) § 403.

For the following listed application(s), please recognize as the "Fee Address" under the provisions of 37 CFR 1.363 the address associated with:

[X] Customer Number: 020016

OR

[ ] The attached Request for Customer Number (PTO/SB/125) form.

PATENT NUMBER (if known)	APPLICATION NUMBER
10,106,603	

Completed by (check one):

[ ] Applicant/Inventor — /Amy McMahon Krom/ (Signature)

[X] Attorney or Agent of record 73,073 (Reg. No.) — Amy McMahon Krom (Typed or printed name)

[ ] Assignee of record of the entire interest. See 37 CFR 3.71. Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96) — 617.646.8000 (Requester's telephone number)

[ ] Assignee recorded at Reel _____ Frame _____ — December 14, 2018 (Date)

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more that one signature is required, see below*.

[ ] * **Total of** 1 **forms are submitted.**

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: December 14, 2018 — Electronic Signature for Brenda M. White: /Brenda M. White/

Electronic Acknowledgement Receipt

EFS ID:	34589300
Application Number:	15988463
International Application Number:	
Confirmation Number:	7597
Title of Invention:	TREATMENT OF FIBROSIS
First Named Inventor/Applicant Name:	Stuart Alexander Cook
Customer Number:	23628
Filer:	Amy McMahon Krom/Brenda White
Filer Authorized By:	Amy McMahon Krom
Attorney Docket Number:	M0546.70012US01
Receipt Date:	14-DEC-2018
Filing Date:	24-MAY-2018
Time Stamp:	12:54:01
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	M054670012US01-TRN-AM.pdf	26029 604ab60c17c101614978c13f26957aa20580fdf1	no	1

Warnings:

Information:					
2	Maintenance Fee Address Change	M054670012US01-CHA-AM.pdf	23763 a68d73dc65865084d2cf2004ea45db892810ec0e	no	1
Warnings:					
Information:					
			Total Files Size (in bytes):	49792	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM *(to be used for all correspondence after initial filing)*		Application Number	Patent#: 10,106,603
		Filing Date	Issued: October 23, 2018
		First Named Inventor	Stuart Alexander Cook
		Art Unit	1646
		Examiner Name	P. M. Mertz
Total Number of Pages in This Submission	2	Attorney Docket Number	M0546.70012US01

ENCLOSURES (*Check all that apply*)

☐ Fee Transmittal Form	☐ Drawing(s)	☐ After Allowance Communication to TC
☐ Fee Attached	☐ Licensing-related Papers	☐ Appeal Communication to Board of Appeals and Interferences
☐ Amendment/Reply	☐ Petition	☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
☐ After Final	☐ Petition to Convert to a Provisional Application	☐ Proprietary Information
☐ Affidavits/declaration(s)	☐ Power of Attorney, Revocation Change of Correspondence Address	☐ Status Letter
☐ Extension of Time Request	☐ Terminal Disclaimer	☒ Other Enclosure(s) (please Identify below):
☐ Express Abandonment Request	☐ Request for Refund	Fee Address Indication Form
☐ Information Disclosure Statement	☐ CD, Number of CD(s) _____	
☐ Certified Copy of Priority Document(s)	☐ Landscape Table on CD	
☐ Reply to Missing Parts/ Incomplete Application	Remarks	
☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53		

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Amy McMahon Krom/		
Printed name	Amy McMahon Krom		
Date	December 14, 2018	Reg. No.	73,073

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: December 14, 2018 Electronic Signature for Brenda M. White: /Brenda M. White/

6837857.1

To: Patents_eOfficeAction@WolfGreenfield.com,WGS_eOfficeAction@WolfGreenfield.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 23628

Oct 04, 2018 04:51:20 AM

Dear PAIR Customer:

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 23628 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:
The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
15988463	ISSUE.NTF	10/03/2018	M0546.70012US01

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/988,463	10/23/2018	10106603	M0546.70012US01	7597

23628 7590 10/03/2018

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206
UNITED STATES OF AMERICA

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Stuart Alexander Cook, Singapore, SINGAPORE;
Singapore Health Services PTE LTD., Singapore, SINGAPORE;
National University of Singapore, Singapore, SINGAPORE;
Sebastian Schaefer, Singapore, SINGAPORE;

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/988,463	05/24/2018	Stuart Alexander Cook	M0546.70012US01	7597

23628 7590 09/25/2018
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206
UNITED STATES OF AMERICA

EXAMINER	
MERTZ, PREMA MARIA	

ART UNIT	PAPER NUMBER
1646	

NOTIFICATION DATE	DELIVERY MODE
09/25/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

Response to Rule 312 Communication	Application No. 15/988,463	Applicant(s)
	Examiner	Art Unit

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address –

1. ☒ The amendment filed on *20 September 2018* under 37 CFR 1.312 has been considered, and has been:

a) ☒ entered.

b) ☐ entered as directed to matters of form not affecting the scope of the invention.

c) ☐ disapproved because the amendment was filed after the payment of the issue fee.
Any amendment filed after the date the issue fee is paid must be accompanied by a petition under 37 CFR 1.313(c)(1) and the required fee to withdraw the application from issue.

d) ☐ disapproved. See explanation below.

e) ☐ entered in part. See explanation below.

CHilliard

To: Patents_eOfficeAction@WolfGreenfield.com,WGS_eOfficeAction@WolfGreenfield.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 23628

Sep 25, 2018 03:35:28 AM

Dear PAIR Customer:

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 23628 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:
The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
15988463	N271	09/25/2018	M0546.70012US01

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

To: Patents_eOfficeAction@WolfGreenfield.com,WGS_eOfficeAction@WolfGreenfield.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 23628

Sep 21, 2018 04:10:24 AM

Dear PAIR Customer:

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 23628 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:
The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
15988463	NTC.PUB	09/20/2018	M0546.70012US01

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/988,463	05/24/2018	Stuart Alexander Cook	M0546.70012US01

CONFIRMATION NO. 7597

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

PUBLICATION NOTICE

Title:TREATMENT OF FIBROSIS

Publication No.US-2018-0265579-A1
Publication Date:09/20/2018

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

Docket No.: M0546.70012US01
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Stuart Alexander Cook
Application No.:	15/988,463
Confirmation No.:	7597
Filed:	May 24, 2018
For:	TREATMENT OF FIBROSIS
Examiner:	P. M. Mertz
Art Unit:	1646

Certificate of Electronic Filing under 37 C.F.R. § 1.8
I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: September 20, 2018 Electronic Signature for Nancy J. Arsenault: /Nancy J. Arsenault/

AMENDMENT AFTER ALLOWANCE UNDER 37 C.F.R. § 1.312

Mail Stop Issue Fee
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

In response to the Notice to File Corrected Application Papers dated September 19, 2017, please amend the above-identified U.S. patent application as follows:

Amendments to the Specification begin on page 2 of this paper.

Remarks/Arguments begin on page 3 of this paper.

AMENDMENTS TO THE SPECIFICATION

Please amend the Specification as follows:

Please replace the paragraph starting on page 92, line 6 with the following replacement paragraph:

We first generated RNA-seq data (Figure 16~~**Error! Reference source not found.**~~) and determined the genotype of 69 ethnically matched (Chinese) individuals in the cohort using a SNP array based on fluorescent probe hybridization supplied by Illumina (HumanOmniExpress 24).

REMARKS

This paper is responsive to the Notice to File Corrected Application Papers dated September 19, 2017 ("Notice"). In the Notice, the Office indicated that page 92, line 6 of the specification submitted on May 24, 2018 contained the phrase "Error! Reference source not found."

By this amendment, Applicant has amended the specification to remove the phrase "Error! Reference source not found." on page 92, line 6. No new matter has been added.

Applicant believes no fee is due with this response. However, if a fee is due, please charge Deposit Account No. 23/2825 under Docket No. M0546.70012US01 from which the undersigned is authorized to draw.

Dated: September 20, 2018

Respectfully submitted,

By /Amy McMahon Krom/
Amy McMahon Krom
Registration No.: 73,073
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

Application No. : 15988463
Applicant : Cook
Filing Date : 05/24/2018
Date Mailed : 09/19/2018

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Notice of Allowance Mailed

This application has been accorded an Allowance Date and is being prepared for issuance. The application, however, is incomplete for the reasons below.

Applicant is given two (2) months from the mail date of this Notice within which to respond. This time period for reply is extendable under 37 CFR 1.136(a) for only TWO additional MONTHS.

The informalities requiring correction are indicated in the attachment(s). If the informality pertains to the abstract, specification (including claims) or drawings, the informality must be corrected with an amendment in compliance with 37 CFR 1.121 (or, if the application is a reissue application, 37 CFR 1.173). Such an amendment may be filed after payment of the issue fee if limited to correction of informalities noted herein. See Waiver of 37 CFR 1.312 for Documents Required by the Office of Patent Publication, 1280 Off. Gaz. Patent Office 918 (March 23, 2004). In addition, if the informality is not corrected until after payment of the issue fee, for purposes of 35 U.S.C. 154(b)(1)(iv), "all outstanding requirements" will be considered to have been satisfied when the informality has been corrected. A failure to respond within the above-identified time period will result in the application being ABANDONED.

See attachment(s).

A copy of this notice MUST be returned with the reply. Please address response to "Mail Stop Issue Fee, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450".

/Quang Nguyen/
Publication Branch
Office of Data Management
(571) 272-4200

Application No. 15988463

IDENTIFICATION OF APPLICATION DEFICIENCIES IN APPLICATION FILED ON OR AFTER SEPTEMBER 16, 2012

[X] Applicant must provide legible text for the following item(s).

- [X] Specification filed 12/16/2016, page(s) 92, line 6 ("Error! Reference source not found.".
- [ ] Claims filed , claim(s) .
- [ ] Other: .

[ ] Applicant must provide missing information on the following page(s) of the specification by amending the specification to add the missing text. No new matter may be added.
Page/line no(s).

[ ] The specification refers to one or more applications by attorney docket number and does not show the U.S. application number(s). Applicant must supply the U.S. application number in place of each attorney docket number.
Page/line no(s).

[ ] Applicant must provide an Abstract of the Disclosure.

[ ] The Application Data Sheet (ADS dated) does not supply the inventor's city and/or does not supply the inventor's U.S. state and/or does not supply the inventor's country. Applicant must submit a signed, in accordance with 37 CFR 1.76(e) and 1.33(b), application data sheet that corrects this deficiency. To be in compliance with 37 CFR 1.76, the corrected application data sheet must identify the information being changed by using underlining for additions and strikethroughs or brackets for deletions.

[ ] Other:

Docket No.: M0546.70012US01
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Stuart Alexander Cook
Application No.:	15/988,463
Confirmation No.:	7597
Filed:	May 24, 2018
For:	TREATMENT OF FIBROSIS
Examiner:	P. M. Mertz
Art Unit:	1646

Certificate of Electronic Filing under 37 C.F.R. § 1.8
I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: September 20, 2018 Electronic Signature for Nancy J. Arsenault: /Nancy J. Arsenault/

RESPONSE TO NOTICE TO CORRECTED APPLICATION PAPERS

Mail Stop Issue Fee
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Notice to File Corrected Application Papers mailed September 19, 2018, Applicant respectfully submits an Amendment After Allowance Under 37 C.F.R. §1.312 and a Copy of the Notice.

Applicant believes no fee is due with this response. However, if a fee is due, please charge Deposit Account No. 23/2825 under Docket No. M0546.70012US01 from which the undersigned is authorized to draw.

Dated: September 20, 2018

Respectfully submitted,

By /Amy McMahon Krom/
Amy McMahon Krom
Registration No.: 73,073
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

6627489.1

Electronic Acknowledgement Receipt

EFS ID:	33776832
Application Number:	15988463
International Application Number:	
Confirmation Number:	7597
Title of Invention:	TREATMENT OF FIBROSIS
First Named Inventor/Applicant Name:	Stuart Alexander Cook
Customer Number:	23628
Filer:	Amy Jeanette McMahon/Nancy Arsenault
Filer Authorized By:	Amy Jeanette McMahon
Attorney Docket Number:	M0546.70012US01
Receipt Date:	20-SEP-2018
Filing Date:	24-MAY-2018
Time Stamp:	11:55:21
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	M054670012US01-TRN-AM.pdf	26346 b37caf7b36f1f8f16f8794c98b50a2023560ad53	no	1

Warnings:

Information:					
2		M054670012US01-312AMN-AM.pdf	30801 9511ea90b0c37038ecc94f6e8cf330da6c1a3562	yes	3

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment after Notice of Allowance (Rule 312)	1	1
	Specification	2	2
	Applicant Arguments/Remarks Made in an Amendment	3	3

Warnings:					
Information:					
3	Miscellaneous Incoming Letter	M054670012US01-NTCAP-AM.pdf	78453 7f6245ef2cf75f5af53beb56c1d385dd1aac38cb	no	2
Warnings:					
Information:					
4	Miscellaneous Incoming Letter	M054670012US01-RNTCAP-AM.pdf	23926 555abd5c2f73d21ed56b594d292e2e4ac8073f76	no	1
Warnings:					
Information:					
		Total Files Size (in bytes):	159526		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM *(to be used for all correspondence after initial filing)*			
		Application Number	15/988,463-Conf. #7597
		Filing Date	May 24, 2018
		First Named Inventor	Stuart Alexander Cook
		Art Unit	1646
		Examiner Name	P. M. Mertz
Total Number of Pages in This Submission		Attorney Docket Number	M0546.70012US01

ENCLOSURES (***Check all that apply***)

☐ Fee Transmittal Form	☐ Drawing(s)	☐ After Allowance Communication to TC
☐ Fee Attached	☐ Licensing-related Papers	☐ Appeal Communication to Board of Appeals and Interferences
☒ Amendment/Reply	☐ Petition	☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
☐ After Final	☐ Petition to Convert to a Provisional Application	☐ Proprietary Information
☐ Affidavits/declaration(s)	☐ Power of Attorney, Revocation Change of Correspondence Address	☐ Status Letter
☐ Extension of Time Request	☐ Terminal Disclaimer	☒ Other Enclosure(s) (please Identify below):
☐ Express Abandonment Request	☐ Request for Refund	Copy of Notice to File Corrected Application Papers Response to Notice to File Corrected Application Papers
☐ Information Disclosure Statement	☐ CD, Number of CD(s) _____	
☐ Certified Copy of Priority Document(s)	☐ Landscape Table on CD	
☐ Reply to Missing Parts/ Incomplete Application	Remarks	
☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53		

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Amy McMahon Krom/		
Printed name	Amy McMahon Krom		
Date	September 20, 2018	Reg. No.	73,073

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: September 20, 2018 Electronic Signature for Nancy J. Arsenault: /Nancy J. Arsenault/

6627427.1

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/988,463	05/24/2018	Stuart Alexander Cook	M0546.70012US01	7597

23628 7590 09/19/2018

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206
UNITED STATES OF AMERICA

EXAMINER	
MERTZ, PREMA MARIA	

ART UNIT	PAPER NUMBER
1646	

NOTIFICATION DATE	DELIVERY MODE
09/19/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

Application No. : 15988463
Applicant : Cook
Filing Date : 05/24/2018
Date Mailed : 09/19/2018

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Notice of Allowance Mailed

This application has been accorded an Allowance Date and is being prepared for issuance. The application, however, is incomplete for the reasons below.

Applicant is given two (2) months from the mail date of this Notice within which to respond. This time period for reply is extendable under 37 CFR 1.136(a) for only TWO additional MONTHS.

The informalities requiring correction are indicated in the attachment(s). If the informality pertains to the abstract, specification (including claims) or drawings, the informality must be corrected with an amendment in compliance with 37 CFR 1.121 (or, if the application is a reissue application, 37 CFR 1.173). Such an amendment may be filed after payment of the issue fee if limited to correction of informalities noted herein. See Waiver of 37 CFR 1.312 for Documents Required by the Office of Patent Publication, 1280 Off. Gaz. Patent Office 918 (March 23, 2004). In addition, if the informality is not corrected until after payment of the issue fee, for purposes of 35 U.S.C. 154(b)(1)(iv), "all outstanding requirements" will be considered to have been satisfied when the informality has been corrected. A failure to respond within the above-identified time period will result in the application being ABANDONED.

See attachment(s).

A copy of this notice MUST be returned with the reply. Please address response to "Mail Stop Issue Fee, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450".

/Quang Nguyen/
Publication Branch
Office of Data Management
(571) 272-4200

Application No. 15988463

IDENTIFICATION OF APPLICATION DEFICIENCIES IN APPLICATION FILED ON OR AFTER SEPTEMBER 16, 2012

[X] Applicant must provide legible text for the following item(s).

- [X] Specification filed 12/16/2016, page(s) 92, line 6 ("Error! Reference source not found.".
- [ ] Claims filed , claim(s) .
- [ ] Other: .

[ ] Applicant must provide missing information on the following page(s) of the specification by amending the specification to add the missing text. No new matter may be added.
Page/line no(s).

[ ] The specification refers to one or more applications by attorney docket number and does not show the U.S. application number(s). Applicant must supply the U.S. application number in place of each attorney docket number.
Page/line no(s).

[ ] Applicant must provide an Abstract of the Disclosure.

[ ] The Application Data Sheet (ADS dated) does not supply the inventor's city and/or does not supply the inventor's U.S. state and/or does not supply the inventor's country. Applicant must submit a signed, in accordance with 37 CFR 1.76(e) and 1.33(b), application data sheet that corrects this deficiency. To be in compliance with 37 CFR 1.76, the corrected application data sheet must identify the information being changed by using underlining for additions and strikethroughs or brackets for deletions.

[ ] Other:

To: Patents_eOfficeAction@WolfGreenfield.com,WGS_eOfficeAction@WolfGreenfield.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 23628

Sep 19, 2018 04:09:22 AM

Dear PAIR Customer:

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 23628 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:
The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
15988463	M327	09/19/2018	M0546.70012US01

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

Nancy J. Arsenault	(Depositor's name)
/Nancy J. Arsenault/	(Signature)
FILED VIA EFS WEB on August 9, 2018	(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/988,463	05/24/2018	Stuart Alexander Cook	M0546.70012US01	7597

TITLE OF INVENTION: TREATMENT OF FIBROSIS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1,000.00	$0	$0	$1,000.00	11/05/2018

EXAMINER	ART UNIT	CLASS-SUBCLASS
Prema Maria Mertz	1646	424-145100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
- [ ] Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- [ ] "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Wolf, Greenfield & Sacks, P.C.
2
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE	(B) RESIDENCE: (CITY and STATE OR COUNTRY)
Singapore Health Services PTE LTD	Singapore, Republic of Singapore
National University of Singapore	Singapore, Republic of Singapore

Please check the appropriate assignee category or categories (will not be printed on the patent) : [ ] Individual [X] Corporation or other private group entity [ ] Government

4a. The following fee(s) are submitted:
- [X] Issue Fee
- [ ] Publication Fee (No small entity discount permitted)
- [ ] Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**
- [ ] A check is enclosed.
- [X] Payment by credit card. ~~Form PTO-2038 is attached~~.
- [X] The Director is hereby authorized to charge ~~the required fee(s),~~ any deficiency, or credit any overpayment, to Deposit Account Number 23/2825 (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
- [ ] Applicant certifying micro entity status. See 37 CFR 1.29 — NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
- [ ] Applicant asserting small entity status. See 37 CFR 1.27 — NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
- [ ] Applicant changing to regular undiscounted fee status. — NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /Amy J. McMahon/ Date August 9, 2018

Typed or printed name Amy J. McMahon, PhD Registration No. 73,073

6523649.1

Electronic Patent Application Fee Transmittal

Application Number:	15988463
Filing Date:	24-May-2018
Title of Invention:	TREATMENT OF FIBROSIS
First Named Inventor/Applicant Name:	Stuart Alexander Cook
Filer:	Amy Jeanette McMahon/Nancy Arsenault
Attorney Docket Number:	M0546.70012US01

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
UTILITY APPL ISSUE FEE	1501	1	1000	1000

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**1000**

Electronic Acknowledgement Receipt	
EFS ID:	33418970
Application Number:	15988463
International Application Number:	
Confirmation Number:	7597
Title of Invention:	TREATMENT OF FIBROSIS
First Named Inventor/Applicant Name:	Stuart Alexander Cook
Customer Number:	23628
Filer:	Amy Jeanette McMahon/Nancy Arsenault
Filer Authorized By:	Amy Jeanette McMahon
Attorney Docket Number:	M0546.70012US01
Receipt Date:	09-AUG-2018
Filing Date:	24-MAY-2018
Time Stamp:	10:46:59
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$1000
RAM confirmation Number	080918INTEFSW10480700
Deposit Account	232825
Authorized User	Wolf Greenfield
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	M054670012US01-TRN-AM.pdf	25043 e54f4104b280a00e9c69c11ec936f47996765af2	no	1
Warnings:					
Information:					
2	Fee Worksheet (SB06)	M054670012US01-FEE-AM.pdf	31497 116a3cf412d6147075628e31b7df43dc889a3162	no	1
Warnings:					
Information:					
3	Issue Fee Payment (PTO-85B)	M054670012US01-ISSFEE-AM.pdf	32940 ed5c1ce369678952bd7db2264d68a9619acbd81f	no	1
Warnings:					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	30353 2ccfd6586cbbd0917ecef09ea162a1bb605cfc7e	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	119833		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM *(to be used for all correspondence after initial filing)*			
		Application Number	15/988,463-Conf. #7597
		Filing Date	May 24, 2018
		First Named Inventor	Stuart Alexander Cook
		Art Unit	1646
		Examiner Name	P. M. Mertz
Total Number of Pages in This Submission		Attorney Docket Number	M0546.70012US01

ENCLOSURES (***Check all that apply***)

[x] Fee Transmittal Form	[ ] Drawing(s)	[ ] After Allowance Communication to TC
[ ] Fee Attached	[ ] Licensing-related Papers	[ ] Appeal Communication to Board of Appeals and Interferences
[ ] Amendment/Reply	[ ] Petition	[ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
[ ] After Final	[ ] Petition to Convert to a Provisional Application	[ ] Proprietary Information
[ ] Affidavits/declaration(s)	[ ] Power of Attorney, Revocation Change of Correspondence Address	[ ] Status Letter
[ ] Extension of Time Request	[ ] Terminal Disclaimer	[x] Other Enclosure(s) (please Identify below):
[ ] Express Abandonment Request	[ ] Request for Refund	Part B - Fee(s) Transmittal
[ ] Information Disclosure Statement	[ ] CD, Number of CD(s) _____	
[ ] Certified Copy of Priority Document(s)	[ ] Landscape Table on CD	
[ ] Reply to Missing Parts/ Incomplete Application	Remarks	
[ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53		

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Amy J. McMahon/		
Printed name	Amy J. McMahon, PhD		
Date	August 9, 2018	Reg. No.	73,073

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: August 9, 2018 Electronic Signature for Nancy J. Arsenault: /Nancy J. Arsenault/

6523658.1

FEE TRANSMITTAL

Complete if known	
Application Number	15/988,463-Conf. #7597
Filing Date	May 24, 2018
First Named Inventor	Stuart Alexander Cook
Examiner Name	P. M. Mertz
Art Unit	1646
Practitioner Docket No.	M0546.70012US01

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29. Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

TOTAL AMOUNT OF PAYMENT ($)1,000.00

METHOD OF PAYMENT (check all that apply)

☐ Check ☒ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

☒ Deposit Account Deposit Account Number: 23/2825 Deposit Account Name: Wolf, Greenfield & Sacks, P.C.

For the above-identified deposit account, the Director is hereby authorized to (check all that apply):

☐ Charge fee(s) indicated below

☐ Charge fee(s) indicated below, **except for the filing fee**

☒ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17, **except for any excess claims fees or multiple dependent claim fee**

☐ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17

☒ Credit any overpayment of fee(s)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES (U = undiscounted fee; S = small entity fee; M = micro entity fee)

	FILING FEES			SEARCH FEES			EXAMINATION FEES			
Application Type	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**Fees Paid ($)**
Utility	300	150*	75	660	330	165	760	380	190	
Design	200	100	50	160	80	40	600	300	150	
Plant	200	100	50	420	210	105	620	310	155	
Reissue	300	150	75	660	330	165	2,200	1,100	550	
Provisional	280	140	70	0	0	0	0	0	0	

* The $150 small entity status filing fee for a utility application is further reduced to $75 for a small entity status applicant who files the application via EFS-Web.

2. EXCESS CLAIM FEES

Fee Description	**Undiscounted Fee ($)**	**Small Entity Fee ($)**	**Micro Entity Fee ($)**
Each claim over 20 (including Reissues)	100	50	25
Each independent claim over 3 (including Reissues)	460	230	115
Multiple dependent claims	820	410	205

Total Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
_____ - 20 or HP =	_____ x	_____ =	_____

HP = highest number of total claims paid for, if greater than 20.

Multiple Dependent Claims	
Fee ($)	**Fee Paid ($)**
_____	_____

Indep. Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
_____ - 3 or HP =	_____ x	_____ =	_____

HP = highest number of independent claims paid for, if greater than 3.

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $400 ($200 for small entity) ($100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	**Extra Sheets**	**Number of each additional 50 or fraction thereof**	**Fee ($)**	**Fee Paid ($)**
_____ - 100 =	_____ /50 =	_____ (round **up** to a whole number) x	_____ =	_____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English specification, $130 fee (no small or micro entity discount)	
Non-electronic filing fee under 37 CFR 1.16(t) for a utility application, $400 fee ($200 small or micro entity)	
Other (e.g., late filing surcharge): 1501 Utility issue fee	1,000.00

SUBMITTED BY

Signature	/Amy J. McMahon/	Registration No. (Attorney/Agent)	73,073	Telephone	617.646.8000
Name (Print/Type)	Amy J. McMahon, PhD			Date	August 9, 2018

Certificate of Electronic Filing under 37 C.F.R. § 1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: August 9, 2018 Electronic Signature for Nancy J. Arsenault: /Nancy J. Arsenault/

6523657.1

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

23628 7590 08/03/2018

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MASSACHUSETTS 02210-2206
UNITED STATES OF AMERICA

EXAMINER	
MERTZ, PREMA MARIA	
ART UNIT	PAPER NUMBER
1646	

DATE MAILED: 08/03/2018

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/988,463	05/24/2018	Stuart Alexander Cook	M0546.70012US01	7597

TITLE OF INVENTION: TREATMENT OF FIBROSIS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	11/05/2018

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

23628 7590 08/03/2018
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MASSACHUSETTS 02210-2206
UNITED STATES OF AMERICA

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/988,463	05/24/2018	Stuart Alexander Cook	M0546.70012US01	7597

TITLE OF INVENTION: TREATMENT OF FIBROSIS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1000	$0.00	$0.00	$1000	11/05/2018

EXAMINER	ART UNIT	CLASS-SUBCLASS
MERTZ, PREMA MARIA	1646	424-145100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. The following fee(s) are submitted:

❑ Issue Fee

❑ Publication Fee (No small entity discount permitted)

❑ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

❑ A check is enclosed.

❑ Payment by credit card. Form PTO-2038 is attached.

❑ The director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

❑ Applicant asserting small entity status. See 37 CFR 1.27

❑ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/988,463	05/24/2018	Stuart Alexander Cook	M0546.70012US01	7597

23628 7590 08/03/2018

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MASSACHUSETTS 02210-2206
UNITED STATES OF AMERICA

EXAMINER	
MERTZ, PREMA MARIA	
ART UNIT	PAPER NUMBER
1646	

DATE MAILED: 08/03/2018

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 15/988,463	Applicant(s) Cook et al.	
	Examiner PREMA M MERTZ	Art Unit 1646	AIA Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to the request submitted on 7/26/18.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 1 and 21-29 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information , please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☑ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☑ All b) ☐ Some *c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☑ Certified copies of the priority documents have been received in Application No. 15/381622 .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file areply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☑ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 6/5/18, 7/13/18, 7/23/18.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☑ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☑ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/PREMA M MERTZ/
Primary Examiner, Art Unit 1646

Notice of Pre-AIA or AIA Status

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

2. Amended claim 1, (5/24/18), and new claims 21-29, (5/24/18), are pending and under consideration by the Examiner.

Claims 2-20 have been canceled.

3. An Examiner's Amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 C.F.R. § 1.312. To ensure consideration of such an amendment, it **MUST** be submitted no later than the payment of the Issue Fee.

Authorization for this Examiner's Amendment was given in a telephone interview with Amy McMahon on 7/26/2018.

4. The application has been amended as follows:

IN THE CLAIMS:

In claim 1, line 2, before “human”, delete "a", substitute therefor --the--.

In claim 1, line 5, delete "signalling”, substitute therefor --signaling--.

5. Claims 1, and 21-29, are allowable.

6. The following is an Examiner's Statement of Reasons for Allowance:

None of the prior art of record describe or suggest a method of treating fibrosis in a human subject, the method comprising administering to the human subject in need of treatment a therapeutically effective amount of an Interleukin 11 receptor α (IL-11Rα)-antibody which is capable of inhibiting Interleukin 11 (IL-11) mediated signalling, wherein the fibrosis is fibrosis of the heart, liver, kidney or eye. The method as recited in the claims is free of the prior art by virtue of the treatment steps and the antibody being administered. Furthermore, the claimed invention meets the requirements of 35 USC § 101 and 35 USC § 112 in that the method provides an alternative method to treat fibrosis of the heart, liver, kidney or eye.

7. Any comments considered necessary by applicant must be submitted no later than the payment of the Issue Fee and, to avoid processing delays, should preferably **accompany** the Issue Fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Advisory Information

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Prema Mertz whose telephone number is (571) 272-0876. The examiner can normally be reached on Monday-Friday from 7:00AM to 3:30PM (Eastern time).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Vanessa Ford, can be reached on (571) 272-0857.

Official papers filed by fax should be directed to (571) 273-8300. Faxed draft or informal communications with the examiner should be directed to (571) 273-0876.

Information regarding the status of an application may be obtained from the Patent application Information Retrieval (PAIR) system. Status information for published applications

may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/PREMA M MERTZ/
Primary Examiner, Art Unit 1646

Examiner-Initiated Interview Summary	Application No. 15/988,463	Applicant(s) Cook et al.	
	Examiner PREMA M MERTZ	Art Unit 1646	AIA Status Yes

All participants (applicant, applicant's representative, PTO personnel):

(1) PREMA MARIA. MERTZ. (3) _____.

(2) Amy McMahon. (4) _____.

Date of Interview: 27 July 2018.

Type: ☑ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☑ No.
If Yes, brief description: _____.

Issues Discussed ☐ 101 ☑ 112 ☐ 102 ☐ 103 ☐ Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: 1 and 21-29.

Identification of prior art discussed: none.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Authorization was obtained to amend claim 1, line 2, to recite "the human subject" to obviate a 35 USC 112(b) rejection.

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/PREMA M MERTZ/ Primary Examiner, Art Unit 1646	

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/988,463	Cook et al.
	Examiner	Art Unit
	PREMA M MERTZ	1646

CPC - Searched*		
Symbol	Date	Examiner
A61K 2039/505	07/26/2018	PM

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
inventor search via NPL, PALM, West	07/26/2018	PM
reviewed parent case 15/381622	07/26/2018	PM
stn/cas search via medline, CAPLUS, USPATFULL	07/26/2018	PM

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
A61K	2039/505	07/26/2018	PM

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/988,463	Cook et al.
	Examiner	**Art Unit**
	PREMA M MERTZ	1646

CPC							
Symbol						**Type**	**Version**
C07K	/	16	/	244		F	2013-01-01
A61K	/	2039	/	54		A	2013-01-01
A61K	/	2039	/	505		A	2013-01-01
C07K	/	2317	/	20		A	2013-01-01
C07K	/	2317	/	76		A	2013-01-01

CPC Combination Sets								
Symbol					**Type**	**Set**	**Ranking**	**Version**
	/		/					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	10	
/PREMA M MERTZ/ Primary Examiner, Art Unit 1646	26 July 2018	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	none

U.S. Patent and Trademark Office — Part of Paper No.: 20180726

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/988,463	Cook et al.
	Examiner	**Art Unit**
	PREMA M MERTZ	1646

INTERNATIONAL CLASSIFICATION					
CLAIMED					
A61K	/	39	/	395	
NON-CLAIMED					
	/		/		

US ORIGINAL CLASSIFICATION	
CLASS	**SUBCLASS**

CROSS REFERENCES(S)						
CLASS	**SUBCLASS (ONE SUBCLASS PER BLOCK)**					

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	10	
/PREMA M MERTZ/ Primary Examiner, Art Unit 1646	26 July 2018	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	none

U.S. Patent and Trademark Office — Part of Paper No.: 20180726

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/988,463	Cook et al.
	Examiner	Art Unit
	PREMA M MERTZ	1646

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIMS

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	1		10		19	9	28								
	2		11		20	10	29								
	3		12	2	21										
	4		13	3	22										
	5		14	4	23										
	6		15	5	24										
	7		16	6	25										
	8		17	7	26										
	9		18	8	27										

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	10	
/PREMA M MERTZ/ Primary Examiner, Art Unit 1646	26 July 2018	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	none

U.S. Patent and Trademark Office — Part of Paper No.: 20180726

WEST Search History

Hide Items | Restore | Clear | Close

DATE: Thursday, July 26, 2018

Hide?	Set Name	Query	Hit Count
DB=PGPB,USPT,EPAB,JPAB,DWPI,FPRS; PLUR=YES; OP=OR			
☐	L8	L6 and L2	5
☐	L7	L6 and L1	5
☐	L6	fibrosis	158919
☐	L5	L3 and l2	5
☐	L4	L3 and L1	5
☐	L3	IL-11 or interleukin-11	19219
☐	L2	Schaefer near Sebastian.in.	94
☐	L1	Cook near Stuart near Alexander.in.	6

END OF SEARCH HISTORY

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 15/988,463	ATTY. DOCKET NO.: M0546.70012US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: May 24, 2018	CONFIRMATION NO.: 7597
				FIRST NAMED INVENTOR: Stuart Alexander Cook	
Sheet	1	of	3	GROUP ART UNIT: 1629	EXAMINER: Not Yet Assigned

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document		Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
		Number	Kind Code		
		2003-0147849	A1	Warne et al.	08-07-2003
		2004-0126358	A1	Warne et al.	07-01-2004
		2004-0142871	A1	Shaughnessy et al.	07-22-2004
		2006-0062760	A1	Keith et al.	03-23-2006
		2007-0160577	A1	Damle et al.	07-12-2007
		2009-0191147	A1	Keith et al.	07-30-2009
		2010-0062058	A1	Warne et al.	03-11-2010
		2010-0093976	A1	Azuma et al.	04-15-2010
		2010-0183544	A1	Jenkins et al.	07-22-2010
		2013-0302277	A1	Jenkins et al.	11-14-2013
		2014-0219919	A1	Edwards et al.	08-07-2014
		2016-0031999	A1	Edwards et al.	02-04-2016
		2017-0174759	A1	Cook et al.	06-22-2017
		5,679,339	A	Keith et al.	10-21-1997
		6,126,933	A	Warne et al.	10-03-2000
		6,540,993	B1	Warne et al.	04-01-2003
		6,846,907	B1	Shaughnessy et al.	01-25-2005
		6,953,777	B1	Keith et al.	10-11-2005
		6,998,123	B1	Shaughnessy et al.	02-14-2006
		7,993,637	B2	Baca	08-09-2011
		8,182,814	B2	Baca et al.	05-22-2012
		8,361,966	B2	Azuma et al.	01-29-2013
		8,518,888	B2	Jenkins et al.	08-27-2013
		8,540,977	B2	Baca	09-24-2013
		9,340,618	B2	Edwards et al.	05-17-2016

FOREIGN PATENT DOCUMENTS

Examiner's Initials #	Cite No.	Foreign Patent Document			Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		Office/ Country	Number	Kind Code			
		WO	1998/36061	A2	The Victoria University of Manchester	08-20-1998	
		WO	2000/078336	A1	Genetics Institute, Inc. et al.	12-28-2000	
		WO	2002/020609	A2	Smithkline Beecham PLC	03-14-2002	
		WO	2005/070446	A1	Scios Inc.	08-04-2005	
		WO	2005/098041	A2	University of Florida Research Foundation, Inc.	10-20-2005	
		WO	2009/052588	A1	CSL Limited	04-30-2009	

EXAMINER:	DATE CONSIDERED:
/PREMA M MERTZ/ (07/25/2018)	07/25/2018

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /P.M.M/

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 15/988,463	ATTY. DOCKET NO.: M0546.70012US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: May 24, 2018	CONFIRMATION NO.: 7597
				FIRST NAMED INVENTOR: Stuart Alexander Cook	
Sheet	2	of	3	GROUP ART UNIT: 1629	EXAMINER: Not Yet Assigned

		WO	2014/121325	A1	CSL Limited	08-14-2014	
		WO	2017/103108	A1	Singapore Health Services PTE, Ltd.	06-22-2017	

OTHER ART – NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)
		International Search Report and Written Opinion for International Patent Application No. PCT/EP2016/081430, mailed April 18, 2017.	
		Chapter II Demand filed August 14, 2017 for International Patent Application No. PCT/EP2016/081430.	
		International Preliminary Report on Patentability (Chapter II) for International Patent Application No. PCT/EP2016/081430, mailed November 6, 2017.	
		Third Party Submission Under 37 C.F.R. § 1.290 for US Application No. 15/381,622 (M0546.70012US00), filed April 30, 2018.	
		ANCEY et al., A fusion protein of the gp130 and interleukin-6Ralpha ligand-binding domains acts as a potent interleukin-6 inhibitor. J Biol Chem. 2003 May 9;278(19):16968-72.	
		BRAVO et al., Crystal structure of a cytokine-binding region of gp130. EMBO J. 1998 Mar 16;17(6):1665-74.	
		CHEN et al., IL-11 receptor alpha in the pathogenesis of IL-13-induced inflammation and remodeling. J Immunol. 2005 Feb 15;174(4):2305-13.	
		DU et al., Interleukin-11: review of molecular, cell biology, and clinical use. Blood. 1997 Jun 1;89(11):3897-908.	
		GARBERS et al., Interleukin-6 and interleukin-11: same same but different. Biol Chem. 2013 Sep;394(9):1145-61. doi: 10.1515/hsz-2013-0166.	
		GU et al., Anti-gp130 transducer monoclonal antibodies specifically inhibiting ciliary neurotrophic factor, interleukin-6, interleukin-11, leukemia inhibitory factor or oncostatin M. J Immunol Methods. 1996 Mar 28;190(1):21-7.	
		HALWANI et al., Airway remodeling in asthma. Curr Opin Pharmacol. 2010 Jun;10(3):236-45. doi: 10.1016/j.coph.2010.06.004.	
		HAM et al., Critical role of interleukin-11 in isoflurane-mediated protection against ischemic acute kidney injury in mice. Anesthesiology. 2013 Dec;l 19(6): 1389-401. doi: 10.1097 I ALN.ObO l 3e3 l 82a950da.	
		KAPINA et al., Interleukin-11 drives early lung inflammation during Mycobacterium tuberculosis infection in genetically susceptible mice. PLoS One. 2011;6(7):e21878. doi: 10.1371/journal.pone.0021878.	
		KHAN et al., Fibrosis in heart disease: understanding the role of transforming growth factor-beta in cardiomyopathy, valvular disease and arrhythmia. Immunology. 2006 May;118(1):10-24.	
		KIMURA et al., Identification of cardiac myocytes as the target of interleukin 11, a cardioprotective cytokine. Cytokine. 2007 May;38(2):107-15.	
		LEE et al., Cysteinyl leukotriene upregulates IL-11 expression in allergic airway disease of mice. J Allergy Clin Immunol. 2007 Jan;119(1):141-9.	
		LEE et al., Endogenous IL-11 signaling is essential in Th2- and IL-13-induced inflammation and mucus production. Am J Respir Cell Mol Biol. 2008 Dec;39(6):739-46. doi: 10.1165/rcmb.2008-0053OC. Epub 2008 Jul 10.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /P.M.M/

EXAMINER:	DATE CONSIDERED:
/PREMA M MERTZ/ (07/25/2018)	07/25/2018

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 15/988,463	ATTY. DOCKET NO.: M0546.70012US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: May 24, 2018	CONFIRMATION NO.: 7597
				FIRST NAMED INVENTOR: Stuart Alexander Cook	
Sheet	3	of	3	GROUP ART UNIT: 1629	EXAMINER: Not Yet Assigned

		LINDAHL et al., Microarray profiling reveals suppressed interferon stimulated gene program in fibroblasts from scleroderma-associated interstitial lung disease. Respir Res. 2013 Aug 2;14:80. doi: 10.1186/1465-9921-14-80.	
		LOKAU et al., Proteolytic Cleavage Governs Interleukin-11Trans-signaling. Cell Rep. 2016 Feb 23;14(7):1761-1773. doi:10.1016/j.celrep.2016.01.053. Epub 2016 Feb 11.	
		LOKAU et al., Signal transduction of Interleukin-11 and Interleukin-6 α-Receptors. Recep Clin Investigation. 2016;3. 5 pages.	
		MINSHALL et al., IL-11 expression is increased in severe asthma: association with epithelial cells and eosinophils. J Allergy Clin Immunol. 2000 Feb;105(2 Pt 1):232-8.	
		MOLET et al., IL-11 and IL-17 expression in nasal polyps: relationship to collagen deposition and suppression by intranasal fluticasone propionate. Laryngoscope. 2003 Oct;113(10):1803-12.	
		MURRAY et al., Targeting interleukin-13 with tralokinumab attenuates lung fibrosis and epithelial damage in a humanized SCID idiopathic pulmonary fibrosis model. Am J Respir Cell Mol Biol. 2014 May;50(5):985-94. doi: 10.1165/rcmb.2013-0342OC.	
		OBANA et al., Therapeutic activation of signal transducer and activator of transcription 3 by interleukin-11 ameliorates cardiac fibrosis after myocardial infarction. Circulation. 2010 Feb 9;121(5):684-91. doi:l0.1161/CIRCULATIONAHA.109.893677.	
		OBANA et al., Therapeutic administration of IL-11 exhibits the postconditioning effects against ischemia-reperfusion injury via STAT3 in the heart. Am J Physiol Heart Circ Physiol. 2012 Sep 1;303(5):H569-77. doi: 10.1152/ajpheart.00060.2012.	
		RAY et al., Regulated overexpression of interleukin 11 in the lung. Use to dissociate development-dependent and -independent phenotypes. J Clin Invest. 1997 Nov 15;100(10):2501-11.	
		SCHAFER et al., IL-11 is a crucial determinant of cardiovascular fibrosis. Nature. 2017 Dec 7;552(7683):110-115. doi: 10.1038/nature24676. Epub 2017 Nov 13.	
		SHEPELKOVA et al., Therapeutic Effect of Recombinant Mutated Interleukin 11 in the Mouse Model of Tuberculosis. J Infect Dis. 2016 Aug 1;214(3):496-501. doi: 10.1093/infdis/jiw176.	
		STANGOU et al., Effect of IL-11 on glomerular expression of TGF-beta and extracellular matrix in nephrotoxic nephritis in Wistar Kyoto rats. J Nephrol. 2011 Jan-Feb;24(1): 106-11.	
		TANG et al., Targeted expression of IL-11 in the murine airway causes lymphocytic inflammation, bronchial remodeling, and airways obstruction. J Clin Invest. 1996 Dec 15;98(12):2845-53.	
		TANG et al., Transforming growth factor-beta stimulates interleukin-11 transcription via complex activating protein-1-dependent pathways. J Biol Chem. 1998 Mar 6;273(10):5506-13.	
		TODA et al., Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. J Allergy Clin Immunol. 2003 Apr;111(4):875-81.	
		TREPICCHIO et al., The therapeutic utility of Interleukin-11 in the treatment of inflammatory disease. Expert Opin Investig Drugs. 1998 Sep;7(9):1501-4.	
		WYNN, Cellular and molecular mechanisms of fibrosis. J Pathol. 2008 Jan;214(2):199-210.	
		YASHIRO et al., Transforming growth factor-beta stimulates interleukin-11 production by human periodontal ligament and gingival fibroblasts. J Clin Periodontol. 2006 Mar;33(3):165-71.	
		ZHU et al., IL-11 Attenuates Liver Ischemia/Reperfusion Injury (IRI) through STAT3 Signaling Pathway in Mice. PLoS One. 2015 May 6;10(5):e0126296. doi: 10.1371/journal.pone.0126296.	

[NOTE – No copies of U.S. patents, published U.S. patent applications, or pending, unpublished patent applications stored in the USPTO's Image File Wrapper (IFW) system, are included. See 37 CFR § 1.98 and 1287OG163. Copies of all other patent(s), publication(s), unpublished, pending U.S. patent applications, or other information listed are provided as required by 37 CFR § 1.98 unless 1) such copies were provided in an IDS in an earlier application that complies with 37 CFR § 1.98, and 2) the earlier application is relied upon for an earlier filing date under 35 U.S.C. § 120.]

EXAMINER:	DATE CONSIDERED:
/PREMA M MERTZ/ (07/25/2018)	07/25/2018

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /P.M.M/

DOCKET NO.: M0546.70012US01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Stuart Alexander Cook
Application No.:	15/988,463
Confirmation No.:	7597
Filed:	May 24, 2018
For:	TREATMENT OF FIBROSIS
Examiner:	Not Yet Assigned
Art Unit:	1629

CERTIFICATE OF ELECTRONIC FILING UNDER 37 C.F.R. § 1.8

The undersigned hereby certifies that this paper, along with any paper referred to as being attached or enclosed, is being transmitted via the Office electronic filing system in accordance with § 1.6(a)(4), on the 5th day of June, 2018.

__/Nathaniel W. Taylor/_____
Nathaniel W. Taylor

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT FILED PURSUANT TO THE DUTY OF DISCLOSURE UNDER 37 C.F.R. §§ 1.56, 1.97 AND 1.98

Sir:

Pursuant to the duty of disclosure under 37 C.F.R. §§ 1.56, 1.97 and 1.98, the undersigned requests consideration of this Information Disclosure Statement.

PART I: Compliance with 37 C.F.R. § 1.97

This Information Disclosure Statement has been filed within three months of the filing date of a national application other than a continued prosecution application under 37 C.F.R. § 1.53(d).

No fee or certification is required.

PART II: Information Cited

The undersigned hereby makes of record in the above-identified application the information listed on the attached form PTO-1449 (modified PTO/SB/08). The order of

/PREMA M MERTZ/ (07/25/2018) 07/25/2018

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /P.M.M/

presentation of the references should not be construed as an indication of the importance of the references.

The undersigned hereby makes the following additional information of record in the above-identified application.

The undersigned would like to bring to the Examiner's attention the following co-pending application that may contain subject matter related to this application:

Serial No.	Filing Date	Inventor(s)	Docket No.
***15/381,622**	**December 16, 2016**	**Cook et al.**	**M0546.70012US00**

*A copy of this reference is not provided as the Office has waived the requirement under 37 C.F.R. § 1.98(a)(2)(iii) for submitting a copy of a cited U.S. patent application if it is scanned to the Image File Wrapper system and is available on Private PAIR.

The undersigned would like to bring to the Examiner's attention the enclosed search report or other communication from a corresponding or related International or Foreign National Application:

Serial No.	Date of Mailing	Type(s) of Communication
PCT/EP2016/081430	**11-06-2017**	**International Preliminary Report on Patentability**
PCT/EP2016/081430	**04-18-2017**	**International Search Report and Written Opinion**

PART III: Remarks

Documents cited anywhere in the Information Disclosure Statement are enclosed unless otherwise indicated. It is respectfully requested that:

1. The Examiner consider completely the cited information, along with any other information, in reaching a determination concerning the patentability of the present claims;

2. The enclosed form PTO-1449 (modified PTO/SB/08) be signed by the Examiner to evidence that the cited information has been fully considered by the United States Patent and Trademark Office during the examination of this application;

3. The citations for the information be printed on any patent which issues from this application.

/PREMA M MERTZ/ (07/25/2018) 07/25/2018

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /P.M.M/

By submitting this Information Disclosure Statement, the undersigned makes no representation that a search has been performed, of the extent of any search performed, or that more relevant information does not exist.

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, in fact, prior art as defined by 35 U.S.C. § 102.

Notwithstanding any statements by the undersigned, the Examiner is urged to form his or her own conclusion regarding the relevance of the cited information.

An early and favorable action is hereby requested.

The Director is hereby authorized to charge any deficiency or credit any overpayment in the fees occasioned by the filing of this Information Disclosure Statement to our Deposit Account No. 23/2825 under Docket No. M0546.70012US01 from which the undersigned is authorized to draw.

Respectfully submitted,

By: /Amy J. McMahon/
Amy J. McMahon, PhD, Reg. No. 73,073
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
Telephone: (617) 646-8000

Docket No.: M0546.70012US01
Date: June 5, 2018
xNDDx

/PREMA M MERTZ/ (07/25/2018) 07/25/2018

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /P.M.M/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

BIB DATA SHEET

CONFIRMATION NO. 7597

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
15/988,463	05/24/2018	424	1646	M0546.70012US01

APPLICANTS

Singapore Health Services PTE LTD., Singapore, SINGAPORE;
National University of Singapore, Singapore, SINGAPORE;

INVENTORS

Stuart Alexander Cook, Singapore, SINGAPORE;
Sebastian Schaefer, Singapore, SINGAPORE;

**** CONTINUING DATA ***************************

This application is a DIV of 15/381,622 12/16/2016 PAT 10035852

**** FOREIGN APPLICATIONS ***************************

UNITED KINGDOM 1522186.4 12/16/2015

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

06/08/2018

Foreign Priority claimed	Met after Allowance	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
☑ Yes ☐ No	☐	SINGAPORE	66	10	1
35 USC 119(a-d) conditions met ☑ Yes ☐ No					
Verified and Acknowledged /PREMA MARIA MERTZ/ Examiner's Signature	Initials				

ADDRESS

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206
UNITED STATES OF AMERICA

TITLE

TREATMENT OF FIBROSIS

FILING FEE RECEIVED	FEES: Authority has been given in Paper	
2120	No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

BIB (Rev. 05/07).

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 15/988,463	ATTY. DOCKET NO.: M0546.70012US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: May 24, 2018	CONFIRMATION NO.: 7597
				FIRST NAMED INVENTOR: Stuart Alexander Cook	
Sheet	1	of	1	GROUP ART UNIT: Not Yet Assigned	EXAMINER: Prema Maria Mertz

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document		Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
		Number	Kind Code		
		2009-0202533	A1	Baca et al.	08-13-2009

FOREIGN PATENT DOCUMENTS

Examiner's Initials #	Cite No.	Foreign Patent Document			Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		Office/ Country	Number	Kind Code			
		KR	20110047179	A		05-06-2011	Y-Abstract & Machine translation

OTHER ART -- NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)
		METZ et al., Characterization of the Interleukin (IL)-6 Inhibitor IL-6-RFP: fused receptor domains act as high affinity cytokine-binding proteins. J Biol Chem. 2007 Jan 12;282(2):1238-48. Epub 2006 Nov 3.	

[NOTE – No copies of U.S. patents, published U.S. patent applications, or pending, unpublished patent applications stored in the USPTO's Image File Wrapper (IFW) system, are included. See 37 CFR § 1.98 and 1287OG163. Copies of all other patent(s), publication(s), unpublished, pending U.S. patent applications, or other information listed are provided as required by 37 CFR § 1.98 unless 1) such copies were provided in an IDS in an earlier application that complies with 37 CFR § 1.98, and 2) the earlier application is relied upon for an earlier filing date under 35 U.S.C. § 120.]

EXAMINER:	DATE CONSIDERED:
/PREMA M MERTZ/ (07/25/2018)	07/25/2018

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

6486069.1

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /P.M.M/

FORM PTO-1449/A and B (modified PTO/SB/08)	APPLICATION NO.: 15/988,463	ATTY. DOCKET NO.: M0546.70012US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT	FILING DATE: May 24, 2018	CONFIRMATION NO.: 7597
	FIRST NAMED INVENTOR: Stuart Alexander Cook	
Sheet 1 of 1	GROUP ART UNIT: 1646	EXAMINER: Prema Maria Mertz Not Yet Assigned

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document Number	Kind Code	Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY

FOREIGN PATENT DOCUMENTS

Examiner's Initials #	Cite No.	Foreign Patent Document Office/ Country	Number	Kind Code	Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		EP	1630232	A2	Conaris research institute AG	03-01-2006	
		WO	1996/019574	A1	Genetics Institute, Inc.	06-27-1996	
		WO	1999/020755	A2	Glaxo Group Limited	04-29-1999	
		WO	2005/058956	A1	Commonwealth Scientific and Industrial Research Organisation	06-30-2005	

OTHER ART -- NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)
		CHOW et al., Structure of an extracellular gp130 cytokine receptor signaling complex. Science. 2001 Mar 16;291(5511):2150-5.	
		JOHNSTONE et al., Emerging roles for IL-11 signaling in cancer development and progression: Focus on breast cancer. Cytokine Growth Factor Rev. 2015 Oct;26(5):489-98. doi: 10.1016/j.cytogfr.2015.07.015. Epub 2015 Jul 14.	
		LEMOLI et al., Interleukin-11 (IL-11) acts as a synergistic factor for the proliferation of human myeloid leukaemic cells. Br J Haematol. 1995 Oct;91(2):319-26.	
		[No Author Listed] Recombinant Human Anti-human Il11 Antibody. Creative Biolabs. 2018 May 8.	
		PUTOCZKI et al., Interleukin-11 is the dominant IL-6 family cytokine during gastrointestinal tumorigenesis and can be targeted therapeutically. Cancer Cell. 2013 Aug 12;24(2):257-71. doi: 10.1016/j.ccr.2013.06.017.	
		SOMMER et al., Constitutively active mutant gp130 receptor protein from inflammatory hepatocellular adenoma is inhibited by an anti-gp130 antibody that specifically neutralizes interleukin 11 signaling. J Biol Chem. 2012 Apr 20;287(17):13743-51. doi: 10.1074/jbc.M111.349167.	

[NOTE – No copies of U.S. patents, published U.S. patent applications, or pending, unpublished patent applications stored in the USPTO's Image File Wrapper (IFW) system, are included. See 37 CFR § 1.98 and 1287OG163. Copies of all other patent(s), publication(s), unpublished, pending U.S. patent applications, or other information listed are provided as required by 37 CFR § 1.98 unless 1) such copies were provided in an IDS in an earlier application that complies with 37 CFR § 1.98, and 2) the earlier application is relied upon for an earlier filing date under 35 U.S.C. § 120.]

EXAMINER:	DATE CONSIDERED:
/PREMA M MERTZ/ (07/25/2018)	07/25/2018

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

6469663.1

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /P.M.M/

To: Patents_eOfficeAction@WolfGreenfield.com,WGS_eOfficeAction@WolfGreenfield.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 23628

Aug 03, 2018 04:00:22 AM

Dear PAIR Customer:

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 23628 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:
The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
15988463	NOA	08/03/2018	M0546.70012US01
	INTV.SUM.EX	08/03/2018	M0546.70012US01
	1449	08/03/2018	M0546.70012US01
	1449	08/03/2018	M0546.70012US01
	1449	08/03/2018	M0546.70012US01
	1449	08/03/2018	M0546.70012US01

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/988,463	05/24/2018	Stuart Alexander Cook	M0546.70012US01	7597

23628 7590 07/26/2018

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206
UNITED STATES OF AMERICA

EXAMINER
MERTZ, PREMA MARIA

ART UNIT	PAPER NUMBER
1646	

NOTIFICATION DATE	DELIVERY MODE
07/26/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Patents_eOfficeAction@WolfGreenfield.com
WGS_eOfficeAction@WolfGreenfield.com

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

In re Application of
Stuart A. Cook, et al.
Application No.: 15/988,463
Filed: May 24, 2018
Attorney Docket No.: M0546.70012US01
For: TREATMENT OF FIBROSIS

: DECISION ON REQUEST TO
: PARTICIPATE IN THE PATENT
: PROSECUTION HIGHWAY
: PROGRAM AND PETITION
: TO MAKE SPECIAL UNDER
: 37 CFR 1.102(a)

This is a decision on the request to participate in the Patent Prosecution Highway (PPH) program and the petition under 37 CFR 1.102(a), filed June 6, 2018, to make the above-identified application special.

The request and petition are **GRANTED**

DISCUSSION

A grantable request to participate in the PPH pilot program and petition to make special require:

1. The U.S. application for which participation in the Global/IP5 PPH pilot program is requested must have the same earliest date, whether this is the priority date or filing date, as that of a corresponding national or regional application filed with another Global/IP5 PPH participating office or a corresponding PCT international application for which one of the Global/IP5 PPH participating offices was the International Searching Authority (ISA) or the International Preliminary Examining Authority (IPEA).

2. Applicant must:
   a. Ensure all the claims in the U.S. application must sufficiently correspond or be amended to sufficiently correspond to the allowable/patentable claim(s) in the corresponding Office of Earlier Examination (OEE) application and
   b. Submit a claims correspondence table in English;

3. Examination of the U.S. application has not begun;

4. Applicant must submit:
    a. Documentation of prior office action:
        i. a copy of the office action(s) just prior to the "Decision to Grant a Patent" from each of the Global/IP5 PPH participating office application(s) containing the allowable/patentable claim(s) or
        ii. if the allowable/patentable claims(s) are from a "Notification of Reasons for Refusal" then the Notification of Reasons for Refusal or
        iii. if the Global/IP5 PPH participating office application is a first action allowance then no office action from the Global/IP5 PPH participating office is necessary should be indicated on the request/petition form or
        iv. the latest work product in the international phase of the OEE PCT application;
    b. An English language translation of the Global/IP5 PPH participating office action or work product from (4)(a)(i)-(ii) or (iv) above;

5. Applicant must submit:
    a. An IDS listing the documents cited by the Global/IP5 PPH participating office examiner in the Global/IP5 PPH participating office action or work product (unless already submitted in this application)
    b. Copies of the documents except U.S. patents or U.S. patent application publications (unless already submitted in this application);

The request to participate in the PPH pilot program and petition comply with the above requirements. Accordingly, the above-identified application has been accorded "special" status.

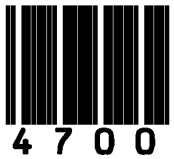
Telephone inquiries concerning this decision should be directed to Angela Walker at (571) 272-1058.

All other inquiries concerning the examination or status of the application is accessible in the PAIR system at http://portal.uspto.gov/.

This application will be forwarded to the examiner for action on the merits commensurate with this decision.

/SDB/
Sherry D. Brinkley
Lead Paralegal Specialist
Office of Petitions

Office of Petitions: Routing Sheet



Application No. 15988463

This application is being forwarded to your office for further processing. A decision has been rendered on a petition filed in this application, as indicated below. For details of this decision, please see the document PET.OP.DEC filed on the same date as this document.

- [X] **GRANTED**
- [ ] **DISMISSED**
- [ ] **DENIED**

Office of Petitions: Decision Count Sheet

Mailing Month: 7

Application No.: 15988463

For US serial numbers: enter number only, no slashes or commas. Ex: 10123456
For PCT: enter "51+single digit of year of filing+last 5 numbers", Ex. for PCT/US05/12345, enter 51512345

Deciding Official: ANGELA, WALKER

Count (1) - Palm Credit 15988463

Decision: GRANT

FINANCE WORK NEEDED
☐ Select Check Box for YES

Decision Type: 652 - Petition to make special-PPH

Notes:

Count (2)

Decision: n/a

FINANCE WORK NEEDED
☐ Select Check Box for YES

Decision Type: NONE

Notes:

Count (3)

Decision: n/a

FINANCE WORK NEEDED
☐ Select Check Box for YES

Decision Type: NONE

Notes:

Initials of Approving Official (if required)

If more than 3 decisions, attach 2nd count sheet & mark this box ☐

Printed on: 7/23/2018

Office of Petitions Internal Document - Ver. 5.0

To: Patents_eOfficeAction@WolfGreenfield.com,WGS_eOfficeAction@WolfGreenfield.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 23628

Jul 26, 2018 04:08:14 AM

Dear PAIR Customer:

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 23628 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:
The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
15988463	PET.OP.DEC	07/26/2018	M0546.70012US01
	PPH.DECISION	07/26/2018	M0546.70012US01

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 15/988,463	ATTY. DOCKET NO.: M0546.70012US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: May 24, 2018	CONFIRMATION NO.: 7597
				FIRST NAMED INVENTOR: Stuart Alexander Cook	
Sheet	1	of	1	GROUP ART UNIT: Not Yet Assigned	EXAMINER: Prema Maria Mertz

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document		Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
		Number	Kind Code		
		2009-0202533	A1	Baca et al.	08-13-2009

FOREIGN PATENT DOCUMENTS

Examiner's Initials #	Cite No.	Foreign Patent Document			Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		Office/ Country	Number	Kind Code			
		KR	20110047179	A		05-06-2011	Y-Abstract & Machine translation

OTHER ART -- NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)
		METZ et al., Characterization of the Interleukin (IL)-6 Inhibitor IL-6-RFP: fused receptor domains act as high affinity cytokine-binding proteins. J Biol Chem. 2007 Jan 12;282(2):1238-48. Epub 2006 Nov 3.	

[NOTE – No copies of U.S. patents, published U.S. patent applications, or pending, unpublished patent applications stored in the USPTO's Image File Wrapper (IFW) system, are included. See 37 CFR § 1.98 and 1287OG163. Copies of all other patent(s), publication(s), unpublished, pending U.S. patent applications, or other information listed are provided as required by 37 CFR § 1.98 unless 1) such copies were provided in an IDS in an earlier application that complies with 37 CFR § 1.98, and 2) the earlier application is relied upon for an earlier filing date under 35 U.S.C. § 120.]

EXAMINER:	DATE CONSIDERED:

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

6486069.1

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 2, pp. 1238–1248, January 12, 2007

Characterization of the Interleukin (IL)-6 Inhibitor IL-6-RFP

*FUSED RECEPTOR DOMAINS ACT AS HIGH AFFINITY CYTOKINE-BINDING PROTEINS**

Received for publication, July 19, 2006, and in revised form, October 17, 2006 Published, JBC Papers in Press, November 3, 2006, DOI 10.1074/jbc.M606885200

Silke Metz[‡], Monique Wiesinger[‡], Michael Vogt[‡], Heike Lauks[§], Günther Schmalzing[§], Peter C. Heinrich[‡], and Gerhard Müller-Newen[‡1]

From the [‡]Institut für Biochemie, Pauwelsstrasse 30 and the [§]Institut für Molekulare Pharmakologie, Universitätsklinikum RWTH Aachen, Wendlingweg 2, 52074 Aachen, Germany

Although fusion proteins of the extracellular parts of receptor subunits termed cytokine traps turned out to be promising cytokine inhibitors for anti-cytokine therapies, their mode of action has not been analyzed. We developed a fusion protein consisting of the ligand binding domains of the IL-6 receptor subunits IL-6Rα and gp130 that acts as a highly potent IL-6 inhibitor. Gp130 is a shared cytokine receptor also used by the IL-6-related cytokines oncostatin M and leukemia inhibitory factor. In this study, we have shown that the IL-6 receptor fusion protein (IL-6-RFP) is a specific IL-6 inhibitor that does not block oncostatin M or leukemia inhibitory factor. We characterized the complex of IL-6-RFP and fluorescently labeled IL-6 (YFP-IL-6) by blue native PAGE and gel filtration. A 2-fold molar excess of IL-6-RFP over IL-6 was sufficient to entirely bind IL-6 in a complex with IL-6-RFP. As shown by treatment with urea and binding competition experiments, the complex of IL-6 and IL-6-RFP is more stable than the complex of IL-6, soluble IL-6Rα, and soluble gp130. By live cell imaging, we have demonstrated that YFP-IL-6 bound to the surface of cells expressing gp130-CFP is removed from the plasma membrane upon the addition of IL-6-RFP. The apparent molecular mass of the IL-6·IL-6-RFP complex determined by blue native PAGE and gel filtration suggests that IL-6 is trapped in a structure analogous to the native hexameric IL-6 receptor complex. Thus, fusion of the ligand binding domains of heteromeric receptors leads to highly specific cytokine inhibitors with superior activity compared with the separate soluble receptors.

Cytokines are important mediators in the regulation of immune responses and inflammation. Dysregulated cytokine signaling leads to chronic inflammation and cancer. Therefore, pro-inflammatory cytokines, such as tumor necrosis factor (TNF)[2] and interleukin-1 and -6, have been identified as promising therapeutic targets. First approaches to specifically block the action of pro-inflammatory cytokines have focused on the use of neutralizing antibodies against a specific cytokine or its receptor. Only recently, the value of soluble cytokine receptors as cytokine antagonists for the treatment of inflammatory diseases has been fully recognized.

Pro-inflammatory cytokines signal through receptor proteins consisting of an extracellular part, a single transmembrane region and a cytoplasmic domain. Ligand binding to the extracellular part of the receptor results in the activation of signal transduction cascades by the cytoplasmic domain. Soluble receptors consisting only of the extracellular part are potent inhibitors of cytokine activity. They bind the cytokine with the same specificity and affinity as the membrane-bound receptors without eliciting an intracellular signal. A dimeric form of the soluble TNF receptor is currently used for the treatment of inflammatory diseases caused by elevated TNF expression (1).

Most cytokines signal through heteromeric receptor complexes consisting of two or more different receptor subunits. In such a case, inhibition of cytokine activity by soluble receptors is more challenging. Recently, we and others have shown that the appropriate fusion of different soluble receptor proteins results in highly potent antagonists (2, 3).

Interleukin-6 (IL-6) has been validated as a target for the treatment of several diseases, such as rheumatoid arthritis and the lymphoproliferative disorder known as Castleman disease (4, 5). IL-6 signals through two different receptor proteins. It first binds to an α-receptor subunit (IL-6Rα). The low affinity complex of IL-6 and IL-6Rα engages the signal transducing receptor subunit gp130 leading to a high affinity receptor complex and the initiation of the cytoplasmic signaling cascades. Upon ligand binding, Janus tyrosine kinases that are constitutively associated with gp130 become activated, resulting in tyrosine phosphorylation of the transcription factor STAT3 (signal transducer and activator of transcription 3). Activated STAT3 accumulates in the nucleus where it induces IL-6 target genes (6).

Soluble gp130 (sgp130) has a moderate antagonistic effect on IL-6 activity (7), whereas soluble IL-6Rα (sIL-6Rα) acts even agonistically (8). Together with IL-6, sIL-6Rα can be regarded as a co-ligand required for the activation of gp130. We have

* This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich SFB 542 and Graduiertenkolleg "Biointerface" GRK 1035) and the Fonds der Chemischen Industrie (Frankfurt am Main). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Institut für Biochemie, Universitätsklinikum RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany. Tel.: 49-241-80-88860; Fax: 49-241-80-2428; E-mail: mueller-newen@rwth-aachen.de.

[2] The abbreviations used are: TNF, tumor necrosis factor; BSA, bovine serum albumin; CBM, cytokine binding module; CFP, cyan fluorescent protein; IL, interleukin; LIF, leukemia inhibitory factor; OSM, oncostatin M; RFP, receptor fusion protein; STAT, signal transducer and activator of transcription; YFP, yellow fluorescent protein; s, soluble; D, domain; R, receptor; ELISA, enzyme-linked immunosorbent assay; Bistris, 2-[bis(2-hydroxyethyl) amino]-2-(hydroxymethyl)propane-1,3-diol.

shown that, in the presence of sgp130, the agonistic activity of sIL-6Rα is converted to an antagonistic activity (9). Thus, the combined application of sIL-6Rα and sgp130 results in a strong inhibition of IL-6 activity.

The extracellular part of gp130 consists of a single Ig-like domain (D1) followed by five fibronectin type III-like domains (D2–D6) (10). Domains D2 and D3 form the cytokine binding module (CBM), which is the structural hallmark of the hematopoietic cytokine receptors (11). The extracellular part of IL-6Rα also consists of an Ig-like domain (D1) followed by a CBM (D2 and D3) (12). By mutational analysis, it was shown that IL-6 contacts its receptors with three distinct binding sites (13). Site I binds the CBM of IL-6Rα (14), and sites II and III interact with the CBM and D1 of gp130, respectively (15).

Recently, the structure of the soluble hexameric IL-6 receptor complex consisting of two molecules of each IL-6, sIL-6Rα (D2–D3), and sgp130 (D1–D3) has been solved by x-ray crystallography (16). One IL-6 molecule contacts with its site II the CBM of one gp130, and with its site III, the Ig-like domain of a second gp130 molecule. The same is valid for the second IL-6 molecule leading to a highly symmetric complex.

Based on the structural features of the IL-6 receptor complex and the well characterized antagonistic activity of the combination of sIL-6Rα and sgp130, we designed a fusion protein (interleukin-6 receptor fusion protein, IL-6-RFP) consisting of the ligand binding domains of gp130 (D1–D3) and IL-6Rα (D2–D3) fused with an appropriate peptide linker (see Fig. 7*A*) (3). We have shown that this fusion protein acts as a highly potent IL-6 inhibitor. The cytokine receptor gp130 is a shared receptor subunit that is used by other IL-6-type cytokines, such as interleukin-11 (IL-11), oncostatin M (OSM), and leukemia inhibitory factor (LIF) (17, 18). Although IL-6-RFP does not inhibit IL-11 (3), its activity toward LIF and OSM has not been tested yet.

Although the importance of fused cytokine receptors as future drugs has been recognized (19), their mode of action has not been analyzed in sufficient detail. Therefore, we investigated the specificity, stability, and stoichiometry of the complex formed by IL-6 and IL-6-RFP. We have shown that IL-6-RFP is a highly specific IL-6 inhibitor that does not interfere with the bioactivity of the related cytokines LIF and OSM. A new method for the analysis of protein-protein interactions based on blue native gel electrophoresis, fluorescent fusion proteins, and fluorescence scanning is presented. We have shown that a 2-fold molar excess of IL-6-RFP over IL-6 is sufficient to completely trap IL-6 in a binary IL-6·IL-6-RFP complex. The complex of IL-6·IL-6-RFP is more stable than the complex of IL-6 with the separate soluble receptors sIL-6Rα and sgp130. As shown in a live cell imaging experiment, YFP-IL-6, once bound to its cell surface receptors, is removed from the plasma membrane upon the addition of IL-6-RFP. Analysis of the stoichiometry by native gel electrophoresis and gel filtration suggests that the architecture of the IL-6·IL-6-RFP complex is analogous to the hexameric receptor complex identified by x-ray crystallography. Thus, IL-6-RFP is a promising IL-6 inhibitor for the treatment of diseases caused by dysregulated IL-6 expression.

EXPERIMENTAL PROCEDURES

Cytokines, Cytokine Receptors—Recombinant human IL-6 was expressed in *Escherichia coli*, refolded, and purified as described previously (20). The specific activity of IL-6 was measured by a B9 cell proliferation assay (21). OSM was purchased from PeproTech (Rocky Hill, NJ) and LIF from Chemicon International (Temecula, CA). sIL-6Rα was expressed in insect cells as previously described (22). sgp130 was obtained from R & D Systems (Minneapolis, MN).

Cell Culture and Transfection of HepG2 Cells—The human hepatoma cell line HepG2 (purchased from the American Type Culture Collection, Manassas, VA) was grown in Dulbecco's modified Eagle's medium F-12 1:1 mix with GlutaMax™I (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Cytogen, Princeton, NJ). For starvation conditions, cells were cultured in pure, serum-free Dulbecco's modified Eagle's medium with 4500 mg/liter glucose, Glutamax™I, and pyruvate (Invitrogen). Plasmids were transiently transfected into HepG2 cells using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions.

Cell Culture and Transfection of COS-7 Cells—The simian monkey kidney cell line COS-7 (kindly provided by I. M. Kerr, Cancer Research UK, London, UK) was cultivated in Dulbecco's modified Eagle's medium with GlutaMax™ (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, 100 mg/liter streptomycin, and 100,000 units/liter penicillin (Cambrex BioScience, Verviers, Belgium). Cells were grown at 37 °C in a water-saturated atmosphere in 5% CO_2. Plasmids were transiently transfected into COS-7 cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol.

Reporter Gene Assay—HepG2 cells were seeded onto 6-well plates (9.6 cm^2/well) and transiently co-transfected with pGL3-α2M-Luc (construct with luciferase gene regulated by the α2 macroglobulin promoter) and pCR™3 LacZ (galactosidase construct with a constitutively active promoter; Amersham Biosciences). Cells were starved in serum-free medium for 6 h and subsequently stimulated with 5 ng/ml IL-6 or the combination of IL-6 and IL-6-RFP for 16 h at the molar ratios indicated. To determine the specificity of IL-6-RFP, HepG2 cells were stimulated in parallel with 0.5 ng/ml OSM or a mixture of OSM and IL-6-RFP, with 5 ng/ml LIF, or a mixture of LIF and IL-6-RFP. Previous to stimulation, the corresponding cytokine and IL-6-RFP were incubated for 30 min at 37 °C to allow complex formation. Preparation of cellular lysates and luciferase measurements were carried out according to the instructions of the manufacturer (Promega). The luciferase activity values were normalized to the transfection efficiency, which was determined as β-galactosidase activity. The experiments were carried out in triplicates, and the mean values and standard deviations were calculated.

Preparation of Cell Lysates, SDS-PAGE, Western Blotting, and Immunodetection—HepG2 cells were cultured on 6-well plates, starved overnight, and stimulated with the indicated cytokine or combination of cytokine and IL-6-RFP for 20 min. Subsequently, cells were lysed with radioimmune precipitation assay lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM NaF, 15% glycerol, 20 mM

β-glycerophosphate, 1 mM Na_3VO_4, 0.25 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 1 μg/ml leupeptin). The lysates were analyzed with SDS-PAGE, Western blotting, and immunodetection using an antibody directed against phosphotyrosine (705)-STAT3 (Cell Signaling Technology, Danvers, MA) or STAT3 (type H190, Santa Cruz Biotechnology, Santa Cruz, CA). Both antibodies were used in a 1:1,000 dilution in TBS-N (20 mM Tris-HCl, pH 7.6, 137 mM NaCl and 0.1% Nonidet P-40).

Purification and Expression of YFP-IL-6 in Insect Cells—High five (H5) insect cells (Invitrogen) were cultured at 27 °C in Sf-900II medium (Invitrogen) containing 2 μg/ml blasticidin. To stably transfect the cells with an expression plasmid coding for YFP-IL-6, the Cellfectin method (Invitrogen) was used. 72 h after seeding of the stably transfected cells, the supernatants were harvested, and subsequently cell debris was removed by centrifugation and sterile filtration. YFP-IL-6 was purified from cell supernatants by affinity chromatography using immobilized sIL-6Rα. The eluates were supplemented with 0.02% bovine serum albumin (BSA) to increase protein stability and dialyzed overnight against phosphate-buffered saline (0.2 M NaCl, 2.5 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4). The concentration of YFP-IL-6 in the eluted fractions was determined by SDS-PAGE, Western blotting, enzyme-linked immunosorbent assay (ELISA) (23), and fluorescence spectroscopy.

Fluorescence Spectroscopy—Samples containing YFP-IL-6 were excited at a wavelength of 514 nm, whereas the light emission between 520 and 600 nm was detected. The emission maximum of YFP at 525 nm was used for the determination of the YFP-IL-6 concentration.

Expression of IL-6-RFP in Baculovirus-infected Insect Cells—IL-6-RFP was cloned as described previously (3). The linker that joins D1–D3 of human gp130 with D2–D3 of human IL-6Rα consists of the flexible extracellular "stalk" region of IL-6Rα (Ala^{323}-Val^{362}) and is therefore supposed to be of low antigenicity. An additional *N*-glycosylation site is introduced with the linker (Asn-Ala-Thr) (3). To produce IL-6-RFP using the baculovirus expression system, the instructions given by the manufacturer were followed. The DNA encoding IL-6-RFP was cut out from the vector pSVL-IL-6-RFP by XbaI and BamHI (Roche Applied Science) and inserted into the polyhedrin locus-based baculovirus transfer vector pVL1392 digested with the same enzymes. Sf21 insect cells were cultivated at 27 °C in Sf-900II medium with 10% fetal calf serum. Cells were co-transfected with 4 μg of pVL1392-IL-6-RFP plasmid and 0.5 μg of BaculoGold™ DNA. Single virus clones were obtained from the cell supernatants by end point dilution. Several clones were screened for expression of IL-6-RFP in Sf21 cells by ELISA. The selected virus clone was then amplified by infecting Sf158 cells cultivated at 27 °C in serum-free Sf-900II medium. For protein expression, exponentially growing Sf158 cells were infected with the recombinant IL-6-RFP baculovirus. Three days after infection, the cell supernatant containing IL-6-RFP (~1 μg/ml) was harvested and cleaned from cells and cellular debris by centrifugation and sterile filtration.

Purification of IL-6-RFP by Affinity Chromatography—IL-6-RFP was purified from Sf158 supernatants by affinity chromatography with IL-6 immobilized to CNBr-Sepharose (Amersham Biosciences). After applying the cell supernatants to the column and washing with phosphate-buffered saline containing 0.05% Tween 20, proteins were eluted with 1 M acetic acid and subsequently neutralized by 2 M Tris-HCl (pH 9). Fractions collected during affinity chromatography were analyzed by SDS-PAGE, silver-staining, Western blotting, and immunodetection with antibodies directed against sIL-6Rα or FLAG (Sigma). The eluates were supplemented with 0.02% BSA (200 μg/ml) and dialyzed overnight against phosphate-buffered saline. The final concentration of purified IL-6-RFP was ~10 μg/ml at a yield of ~70%. IL-6-RFP was stored for up to three years at −20 °C without major loss of activity.

Quantification of IL-6-RFP by ELISA—An ELISA for the quantification of FLAG-tagged IL-6-RFP was carried out as described previously (9). The ELISA plates were coated with 0.3 μg/ml FLAG monoclonal antibody (Sigma), and 50 ng/ml biotinylated monoclonal antibody B-T2 (Diaclone, Besançon, France) was used as the secondary antibody. The standard curve was obtained by 2-fold serial dilutions of sgp130-FLAG expressed in COS-7 cells and calibrated by sgp130 ELISA (9).

Blue Native PAGE and Detection of Fluorescence of YFP—To allow complex formation, YFP-IL-6 and IL-6-RFP (or YFP-IL-6, sIL-6Rα, and sgp130) were incubated for 30 min at room temperature at molar ratios as indicated. Complexes were separated by blue native PAGE (24, 25) using gradient polyacrylamide gels (from 4 to 12 or 4 to 20% polyacrylamide). The cathode buffer was composed of 50 mM tricine, 15 mM Bistris, and 0.002% Coomassie Brilliant Blue® G 250 (Serva, Heidelberg, Germany), whereas the anode buffer contained 50 mM Bistris/HCl, pH 7. BSA was used as molecular weight marker protein. In addition, BSA was utilized as an internal marker protein, because YFP-IL-6 and IL-6-RFP were supplemented with BSA to increase protein stability. The fluorescence of YFP was detected with a Typhoon gel imager (Amersham Biosciences) using an excitation at 488 nm, whereas the emission was detected using a 500–540 nm band-pass filter. After detection, the gels were fixed and silver-stained.

Live Cell Imaging by Confocal Laser Scanning Microscopy—Confocal microscopy of living cells was carried out using a Zeiss LSM 510 Meta confocal laser-scanning microscope (Zeiss, Jena, Germany) equipped with an argon laser, a 63×/1.2 aperture water-corrected objective, an open cell cultivation chamber (Pecon, Erbach, Germany), and a CO_2 incubation and heating unit (Zeiss). For live cell imaging, COS-7 cells were transfected with pSVL-gp130id-CFP coding for an internalization-deficient mutant of gp130 fused to CFP (26). Subsequently, cells were seeded onto 42-mm glass coverslips, and 48 h after transfection, coverslips were placed into the open cell cultivation chamber. The CO_2 incubation and heating unit maintained a constant CO_2 amount of 5% and a temperature of 37 °C. For recording of multiple fluorescence signals from one cell, the multitrack function of the LSM 510 Meta microscope was used. Cyan fluorescence was excited with λ = 458 nm (5% transmission) and detected after a band-pass filter BP 480/20. Yellow fluorescence was excited with λ = 514 nm (5% transmission) and a band-pass filter BP 530–600 was used. All images represent confocal slices of ~1 μm. Cells were co-stimulated with 240 ng/ml YFP-IL-6 and 3 μg/ml sIL-6Rα, and CFP and YFP

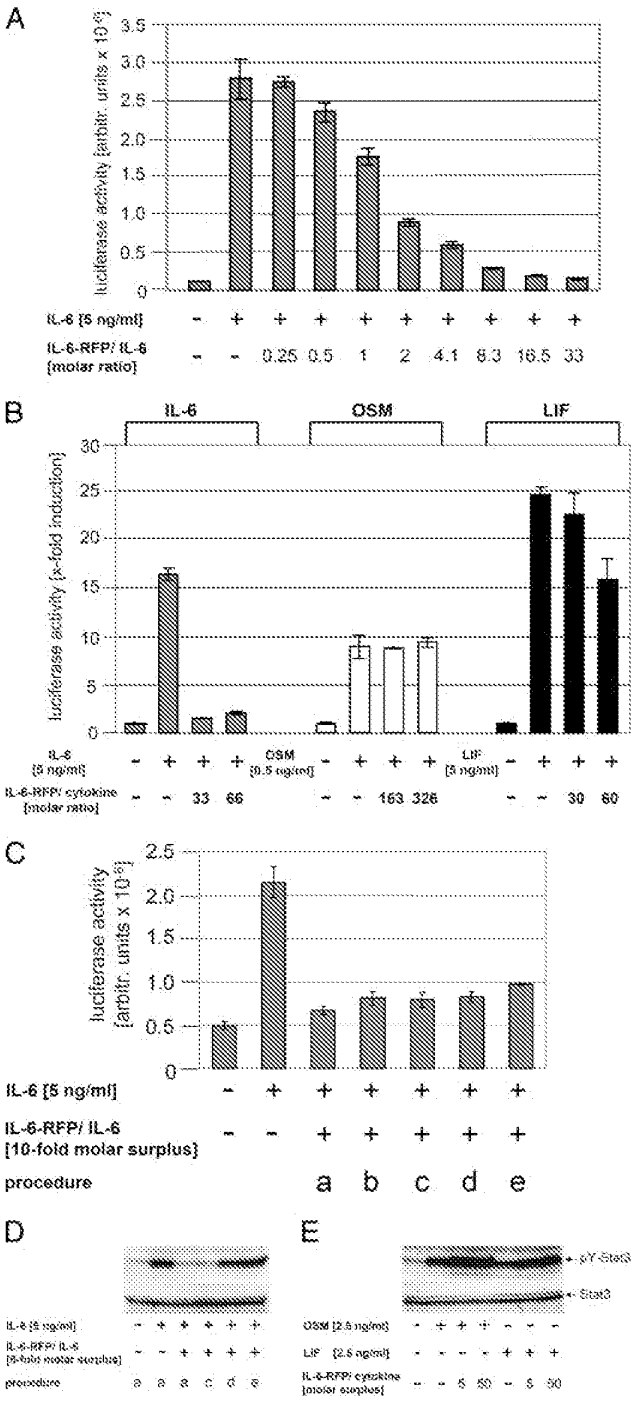


FIGURE 1. **Bioactivity and specificity of IL-6-RFP.** *A*, HepG2 cells were transfected with a reporter gene plasmid coding for luciferase under the control of the IL-6-responsive rat α2M promoter. To investigate the concentration dependence of the inhibition of the IL-6-induced luciferase activity by IL-6-RFP, IL-6 and IL-6-RFP were incubated at the indicated molar ratios for 30 min at 37 °C to allow complex formation. Subsequently, HepG2 cells were stimulated for 16 h with the mixture of cytokine and inhibitor. Luciferase activity was measured in triplicates. *B*, HepG2 cells were transfected as described for *A* and stimulated with IL-6, OSM, or LIF. To investigate the specificity of the IL-6 inhibitor, IL-6, OSM, or LIF were preincubated with IL-6-RFP for 30 min at 37 °C at the molar ratios indicated. Subsequently, HepG2 cells were stimulated for 16 h with the mixture of cytokine and inhibitor, and the luciferase activity induced upon the α2M promoter activation was measured. *C*, HepG2 cells were transfected as described for *A*. IL-6 was incubated with a 10-fold molar excess of IL-6-RFP for 30 min at 37 °C. Afterward, HepG2 cells were stimulated for 16 h with the mixture of cytokine and inhibitor (*procedure a*). In *procedure b*, HepG2 cells were preincubated with IL-6-RFP for 30 min and then stimulated with IL-6 for 16 h, whereas in *procedure c*, IL-6 and IL-6-RFP were added simultaneously to the cells without preincubation. In *procedures d* and *e*, HepG2 cells were stimulated with IL-6 for 10 or 20 min, respectively, and subsequently treated with IL-6-RFP for 16 h. After that time, luciferase activity induced upon α2M promoter activation was measured. *D*, HepG2 cells were seeded onto 6-well plates and serum-starved overnight. Subsequently, IL-6 was incubated with a 5-fold molar surplus of IL-6-RFP at 37 °C for 30 min to allow complex formation. The cells were stimulated with IL-6 or the mixture of IL-6 and IL-6-RFP for 20 min (*procedure a*). In *procedure c*, IL-6 and IL-6-RFP were added simultaneously to the cells (without preincubation) for 20 min. In *procedures d* and *e*, HepG2 cells were stimulated with IL-6 for 10 or 20 min, respectively, and afterward treated with IL-6-RFP for 20 min. *E*, OSM and IL-6-RFP or LIF and IL-6-RFP were preincubated for 30 min at 37 °C at the molar ratios indicated. HepG2 cells were stimulated with OSM or LIF or a mixture of OSM and IL-6-RFP or LIF and IL-6-RFP for 20 min. After stimulation, HepG2 cells were lysed. The lysates were analyzed by SDS-PAGE, Western blot, and immunodetection with phospho-STAT3- or STAT3-specific antibodies.

fluorescence was measured every 4 min over 1 h. In a second approach, a 10-fold molar excess of IL-6-RFP over YFP-IL-6 was added to the cells 21 min after co-stimulation.

Gel Filtration—Gel filtration was carried out at 4 °C with a Superdex 200 16/60 column (Amersham Biosciences) at a flow rate of 1 ml/min. During each run, the light absorption at 280 nm was measured. The column was calibrated with 9 mg of a gel filtration standard protein mixture containing thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa) (Bio-Rad). During gel filtration of YFP-IL-6 and the YFP-IL-6·IL-6-RFP complex, 3-ml fractions were collected.

RESULTS

IL-6-RFP Inhibits Acute Phase Protein Gene Induction and STAT3 Activation by IL-6 but Not by the Related Cytokines OSM and LIF—Human hepatocellular carcinoma cells (HepG2) respond to IL-6, OSM, and LIF stimulation with the induction of acute phase protein genes (27) and are therefore well suited to test the specificity of IL-6-RFP. As shown in Fig. 1*A*, IL-6-RFP concentration-dependently inhibited the induction of the α2 macroglobulin (α2M) promoter by IL-6 in a reporter gene assay. A 2-fold molar excess of IL-6-RFP over IL-6 led to a reduction of the biological response of ~70%. A >10-fold molar excess suppressed gene induction to nearly basal levels. At concentrations of IL-6-RFP, which completely inhibited IL-6 activity, gene induction by OSM was not disturbed (Fig. 1*B*). At a 30-fold molar excess of IL-6-RFP over LIF, LIF activity was not affected. A moderate inhibition of LIF activity was observed at a 60-fold excess. Fig. 1*C* shows that IL-6-RFP acts on IL-6-mediated reporter gene induction not only after preincubation of the cytokine and the inhibitor (*procedure a*) but also when the inhibitor is given simultaneously (*procedures b* and *c*) or with a delay of 10 min (*procedure d*) or 20 min (*procedure e*). Reporter gene induction was measured 16 h after cytokine stimulation, whereas STAT3 tyrosine phosphorylation could be detected within minutes (Fig. 1*D*). STAT3 activation was inhibited when IL-6 and IL-6-RFP were preincubated for 30 min (*procedure a*) or added simultaneously (*procedure c*). In this short term assay, a delay of

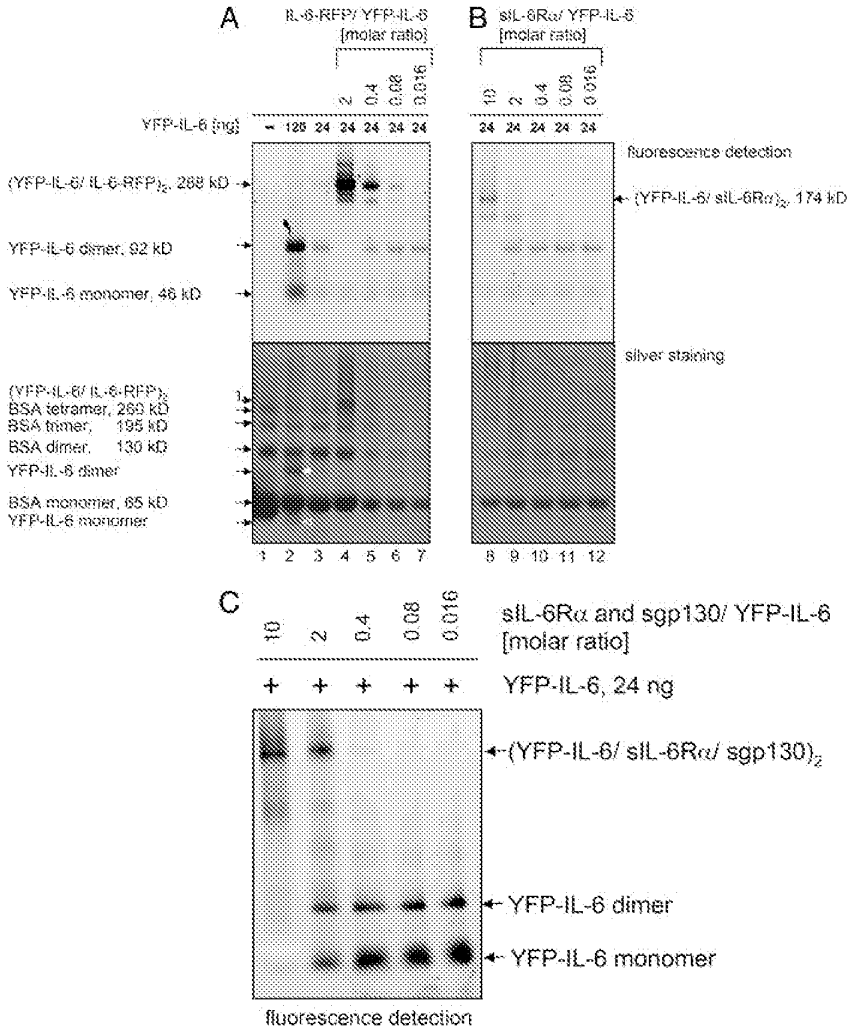


FIGURE 2. **Blue native PAGE; YFP-IL-6 binds to IL-6-RFP with a higher affinity than to sIL-6Rα or the combination of sIL-6Rα and sgp130.** To compare the concentrations of IL-6-RFP (*A*), sIL-6Rα (*B*), or the combination of sIL-6Rα and sgp130 (*C*) sufficient to bind to YFP-IL-6, YFP-IL-6 was incubated with a 2-, 0.4-, 0.08- or 0.016-fold molar ratio of IL-6-RFP for 30 min at room temperature. In a parallel approach, YFP-IL-6 was mixed with a 10-, 2-, 0.4-, 0.08-, or 0.016-fold molar ratio of sIL-6Rα or a 10-, 2-, 0.4-, 0.08-, or 0.016-fold molar ratio of the combination of sIL-6Rα and sgp130 and also incubated for 30 min at room temperature. The protein complexes were separated by blue native PAGE, and the fluorescent YFP-IL-6 was visualized by fluorescence detection. Subsequently, the polyacrylamide gels were fixed and silver-stained.

the action of IL-6-RFP was not tolerated (*procedures d* and *e*). IL-6-RFP did not interfere with STAT3 activation induced by LIF or OSM (Fig. 1*E*).

Analysis of the Interaction between IL-6 and IL-6-RFP by a Fluorescence Gel Shift Assay—Although the bioactivity of IL-6-RFP has been analyzed in detail (Fig. 1) (3), the complex formation of IL-6-RFP and IL-6 has not been studied. In a recent publication, we have shown that fusion of the yellow fluorescent protein (YFP) to the N terminus of IL-6 does not affect its bioactivity (26). The fluorescent cytokine YFP-IL-6 was expressed in insect cells and purified by affinity chromatography. For stabilization of diluted solutions of YFP-IL-6, BSA was added. Different amounts of YFP-IL-6/BSA were loaded onto a non-denaturing polyacrylamide gradient gel, and electrophoresis was performed under blue native PAGE conditions. When the wet gel was analyzed by a fluorescence scanner, YFP-IL-6 was readily detectable as two separate bands (Fig. 2*A*, *upper panel*, *lanes 2* and *3*).

Subsequently, the same gel was silver-stained (Fig. 2*A*, *lower panel*). At the higher concentration (120 ng), the upper YFP-IL-6 band is clearly visible (Fig. 2*A*, *filled triangle*, *lane 2*), whereas the lower one is only weakly stained (*open triangle*, *lane 2*). At the lower concentration (24 ng) (Fig. 2*A*, *lane 3*), YFP-IL-6 is hardly visible. Thus, the sensitivity of the fluorescence detection was similar or even exceeded the sensitivity of a silver stain. In *lane 1*, albumin alone was loaded onto the gel. The albumin monomer (65 kDa) is the strongest band, but the dimer (130 kDa), trimer (195 kDa), and tetramer (260 kDa) are also clearly detectable. These bands serve as molecular mass markers. The calculated molecular mass of YFP-IL-6 is 46 kDa. Therefore, the band between the albumin dimer (130 kDa) and the albumin monomer (65 kDa) corresponds to a dimer of YFP-IL-6 (92 kDa), whereas the lower band represents the monomer (46 kDa).

The addition of IL-6-RFP to YFP-IL-6 resulted in a marked shift of the fluorescent band, which indicates complex formation of YFP-IL-6 with IL-6-RFP (Fig. 2*A*, *lanes 4–6*). We often observed that the band shift was accompanied with a considerable increase in fluorescence intensity. We attribute this phenomenon to quenching or dequenching of the fluorescence, dependent on varying local concentrations of Coomassie, salts, and polyacrylamide in the gradient gel. A 2-fold molar excess of IL-6-RFP was sufficient to trap YFP-IL-6 completely (*lane 4*). Still at a 0.4- or 0.08-fold molar ratio of IL-6-RFP over YFP-IL-6, a complex was formed. To achieve a complex formation between sIL-6Rα and YFP-IL-6, a 10-fold molar excess of sIL-6Rα is required (Fig. 2*B*, *lane 8*). The complex already disappears at a 2-fold molar excess (Fig. 2*B*, *lane 9*, compare with *lane 4*). The combination of sIL-6Rα and sgp130 (Fig. 2*C*) required a 10-fold molar excess to completely bind YFP-IL-6, whereas a 2-fold molar excess led to only a partial trapping of YFP-IL-6 (Fig. 2*A*, compare with *lane 4*). These results indicate that IL-6-RFP binds YFP-IL-6 more efficiently than its soluble receptors.

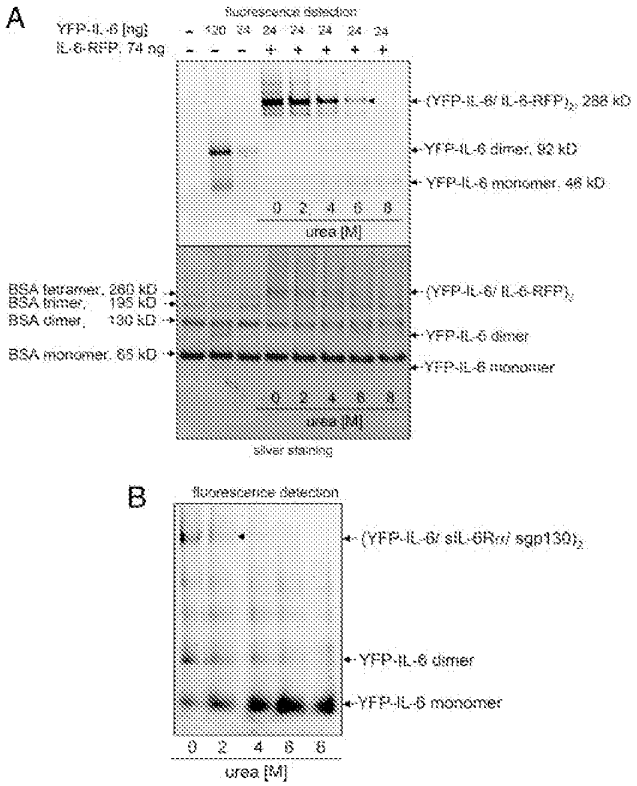


FIGURE 3. **Blue native PAGE; analysis of the stability of the complex formed by IL-6-RFP and YFP-IL-6 and the complex of YFP-IL-6·sIL-6Rα·sgp130 in the presence of urea.** To allow complex formation, 24 ng of YFP-IL-6 were incubated with a 2-fold molar surplus of IL-6-RFP (74 ng) for 30 min at room temperature (*A*). In a parallel approach (*B*), YFP-IL-6 (24 ng), sIL-6Rα (44 ng), and sgp130 (74 ng) were mixed at a molar ratio of 1:2:2 and incubated at room temperature for 30 min to enable complex formation. Thereafter, the indicated amounts of urea were added and incubated for another 10 min at room temperature. After incubation, the protein complexes were separated by blue native PAGE, and the fluorescent YFP-IL-6 was visualized by fluorescence detection. Subsequently, the polyacrylamide gel was fixed and silver-stained.

IL-6 Forms a More Stable Complex with IL-6-RFP than with Its Soluble Receptors—Three experiments were performed to assess the stability of the complexes formed by IL-6 and IL-6-RFP or IL-6 and its soluble receptors. In a first experiment, the complexes were incubated in the presence of urea at increasing concentrations. Although the complex of IL-6·IL-6-RFP is still detectable in the presence of 6 M urea (Fig. 3*A*, *filled triangle*), only a faint band of the IL-6·sIL-6Rα·sgp130 complex is visible in the presence of 2 M urea (Fig. 3*B*, *filled triangle*), which disappears at a concentration of 4 M urea. Interestingly, with increasing concentrations of urea, the band of the YFP-IL-6 monomer becomes more intense (Fig. 3), suggesting that urea induces a dissociation of the YFP-IL-6 dimers into monomers.

In a second approach, the complexes formed by YFP-IL-6 and IL-6-RFP or YFP-IL-6 and its soluble receptors were incubated with varying amounts of unlabeled IL-6 for 2 h to determine the replacement of YFP-IL-6 by IL-6. A 10-fold molar excess of IL-6 over YFP-IL-6 was required to detect a replacement of YFP-IL-6 in the complex of YFP-IL-6 and IL-6-RFP (Fig. 4*A*). The fluorescence intensity of the complex decreased at a 10-fold molar excess of IL-6 over YFP-IL-6 (Fig. 4*A*, *filled triangle*), whereas the fluorescence of the YFP dimer became more intense (*open triangle*). In the case of the receptor complex formed by YFP-IL-6 and its soluble receptors, lower amounts of unlabeled IL-6 were sufficient to displace YFP-IL-6 (Fig. 4*B*). The fluorescence of the YFP-IL-6 dimer increased when IL-6 was added at a 0.1-fold molar ratio (Fig. 4*B*, *open triangle*).

In a complementary third approach, increasing amounts of sIL-6Rα and sgp130 were added to the complex formed by YFP-IL-6 and IL-6-RFP (Fig. 4*C*). Hardly any YFP-IL-6·sIL-6Rα·sgp130 ternary complex was detectable after 2 h. Conversely, when IL-6-RFP was added to a preformed YFP-IL-6·sIL-6Rα·sgp130 ternary complex, the complex of YFP-IL-6·IL-6-RFP was formed within 2 h at only a 0.1-fold molar concentration of IL-6-RFP over sIL-6Rα and sgp130 (Fig. 4*D*). Thus, the cytokine is more efficiently trapped in complex with IL-6-RFP than in complex with the soluble receptors.

IL-6-RFP Removes YFP-IL-6 from the Cell Surface Receptor Complex—Next, we asked whether IL-6-RFP, as a result of its superior activity, would be able to remove IL-6 from its receptor on the plasma membrane. Therefore, we transfected COS-7 cells with an internalization-deficient (id) mutant of gp130 fused to CFP (gp130id-CFP (26)) and analyzed living cells by confocal laser scanning microscopy. As shown in Fig. 5*A*, besides endoplasmic reticulum and Golgi apparatus, the plasma membrane of transfected cells is clearly visible in the CFP channel resulting from surface expression of gp130id-CFP. After the addition of YFP-IL-6 and sIL-6Rα (COS-7 cells lack endogenous IL-6Rα), the fluorescent cytokine accumulated at the cell surface resulting in a membrane staining detected in the YFP channel. The intensities of YFP and CFP fluorescence in the regions of interest at the plasma membrane (Fig. 5*A*, *red rectangles*) are depicted in Fig. 5*B*. YFP-IL-6 binding to gp130id-CFP reached saturation within 50 min (Fig. 5*B*, *left panel*).

In a second experiment (Fig. 5*A*, *right panel*), a 10-fold molar excess of IL-6-RFP over YFP-IL-6 was added 21 min after YFP-IL-6/sIL-6Rα stimulation when YFP-IL-6 binding was near saturation. The addition of IL-6-RFP resulted in a decrease of cell surface staining of YFP-IL-6, as shown in the image of the YFP channel 49 min after stimulation. The corresponding fluorescence intensities of YFP and CFP in the indicated region of interest are presented in the *right diagram* of Fig. 5*B*. After the addition of IL-6-RFP, the YFP-IL-6 fluorescence decreased, whereas gp130id-CFP fluorescence remained constant. The decrease of the YFP/CFP ratio at the plasma membrane indicates that YFP-IL-6 is removed from the cell surface. Within 25 min, the IL-6-occupied receptors decreased by 50%. Thus, IL-6-RFP has the capacity to eliminate IL-6 from activated receptor complexes at the cell surface.

Determination of the Molecular Mass of the YFP-IL-6·IL-6-RFP Complex by Gel Filtration—IL-6-RFP was purified from insect cell supernatants by affinity chromatography on IL-6-Sepharose (Fig. 6*A*). In non-reducing SDS-PAGE, IL-6-RFP appears as a monomeric protein with an apparent molecular mass of ~80 kDa (Fig. 6*A*, *lane 2*). We used a calibrated gel filtration column to determine the molecular masses of YFP-IL-6, IL-6-RFP, and the complex of YFP-IL-6 and IL-6-RFP. YFP-IL-6 was detected by fluorescence spectroscopic analysis

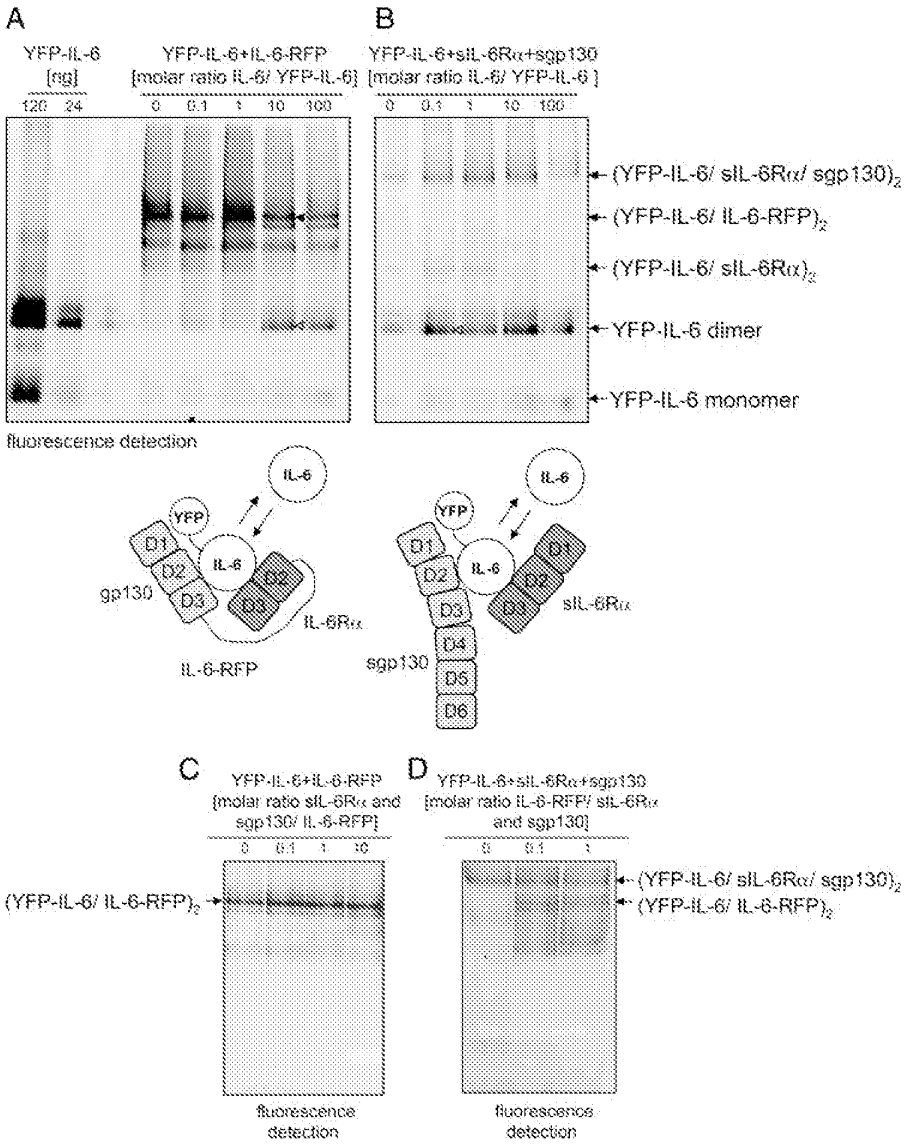


FIGURE 4. **Blue native PAGE; competition of IL-6 with YFP-IL-6 trapped by IL-6-RFP or by sIL-6Rα and sgp130.** *A*, YFP-IL-6 was incubated with a 2-fold molar surplus of IL-6-RFP for 30 min at room temperature to allow complex formation. In a parallel approach (*B*), YFP-IL-6 was mixed with a 2-fold molar surplus of sIL-6Rα and sgp130. Subsequently, non-tagged IL-6 was added at increasing concentrations (0-, 0.1-, 1-, 10-, and 100-fold molar ratio over YFP-IL-6) and incubated for 2 h at room temperature. The protein complexes were separated by blue native PAGE, and the fluorescent YFP-IL-6 was visualized by fluorescence detection. The complexes YFP-IL-6·IL-6-RFP (*C*) and YFP-IL-6·sIL-6Rα·sgp130 (*D*) were preformed by incubation at 37 °C for 30 min at molar ratios 1:2 (*C*) or 1:10:10 (*D*). Subsequently, the complex YFP-IL-6·IL-6-RFP was incubated with a 0-, 0.1-, 1-, and 10-fold molar excess of sIL-6Rα and sgp130 over IL-6-RFP for 2 h (*C*), and the complex YFP-IL-6·sIL-6Rα·sgp130 was incubated with a 0-, 0.1-, 1-fold molar ratio of IL-6-RFP over the soluble receptors for 2 h (*D*).

of the collected fractions. IL-6-RFP was quantified by ELISA (3). YFP-IL-6 alone eluted in two peaks from the gel filtration column (Fig. 6*B*). According to the retention times, these peaks correspond to YFP-IL-6 monomers (47 kDa) and YFP-IL-6 dimers (94 kDa). This finding is in line with the two species detected by blue native PAGE.

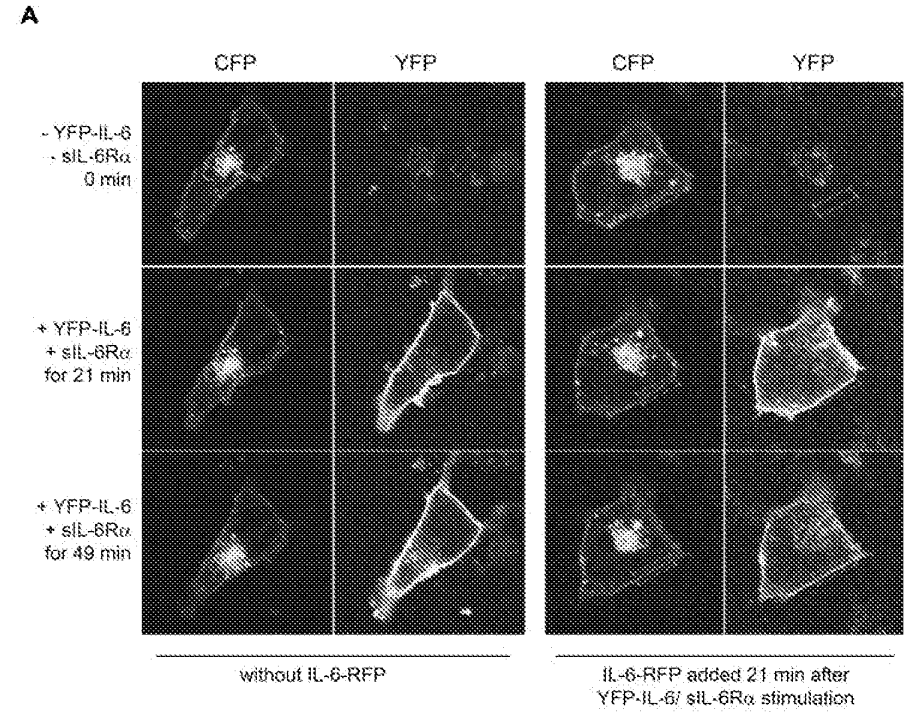
The elution profile of IL-6-RFP is shown in Fig. 6*C*. Interestingly, no monomeric IL-6-RFP could be detected. The major portion of IL-6-RFP is found in a peak corresponding to 170 kDa that most probably represents the IL-6-RFP dimer. Also, some higher molecular mass species were detected.

Pre-incubation of YFP-IL-6 with a 2-fold molar excess of IL-6-RFP resulted in a dramatic change in the elution patterns (Fig. 6*D*). The whole population of YFP-IL-6 eluted earlier from the column corresponding to a higher apparent molecular mass. This finding indicates that YFP-IL-6 is completely trapped in a complex with IL-6-RFP, confirming the results obtained by blue native PAGE. Relevant fractions were analyzed by ELISA to detect IL-6-RFP (Fig. 6*D*, *blue line*). All fractions that contained the shifted YFP-IL-6 also contained IL-6-RFP. In addition, a second peak of IL-6-RFP devoid of YFP-IL-6 appeared. This peak corresponds to the unliganded IL-6-RFP dimer.

The apparent molecular mass of the YFP-IL-6·IL-6-RFP complex derived from gel filtration (Fig. 6*D*) is 325 kDa. If the complex is built up according to the native hexameric receptor complex (consisting of two molecules each of IL-6, IL-6Rα, and gp130 (16)), a tetrameric complex is expected, consisting of two molecules of YFP-IL-6 and two molecules of IL-6-RFP. The calculated molecular mass for such a complex is 264 kDa. The discrepancy between the observed and calculated values can be attributed to the bulky architecture of the IL-6 receptor complex (16) as discussed below.

DISCUSSION

According to the x-ray structure of the soluble IL-6 receptor complex, IL-6 signals through a hexameric complex consisting of two molecules each of IL-6, IL-6Rα, and gp130 (16). The soluble receptors sIL-6Rα and sgp130 are present in human blood in considerable concentrations (50 ng/ml (28, 29) and 300 ng/ml (7, 9), respectively). We found that the pairwise action of these soluble receptors blocks systemic IL-6 responses (9). Based on this observation, we designed a fusion protein composed of the ligand binding domains of IL-6Rα (Fig. 7*A*, *D2* and *D3*) and gp130 (*D1–D3*), which turned out to be a highly potent inhibitor of IL-6 (3). Such novel cytokine inhibitors are promising candidates for future drugs against



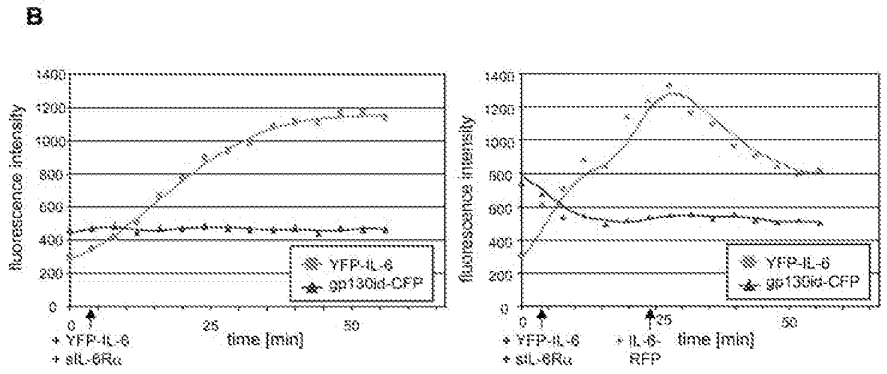


FIGURE 5. **Live cell imaging of removal of YFP-IL-6 from the surface of gp130id-CFP-transfected cells by IL-6-RFP.** *A*, COS-7 cells were transfected with 4 μg of pSVL-gp130id-CFP, seeded onto coverslips, and investigated in an open cell cultivation chamber by confocal laser scanning microscopy. Cells were stimulated with 240 ng/ml YFP-IL-6 and 3 μg/ml sIL-6Rα, and pictures were taken every 4 min over 1 h (*left panel*). In a second approach (*right panel*), a 10-fold molar excess of IL-6-RFP was added to the cells 21 min after stimulation. The region of interest used for quantitative evaluation in *B* is marked in *red*. The cells shown are representative of seven cells in each approach. *B*, quantitative evaluation of *A* using the membrane region of interest indicated in each picture. The *curve* showing the CFP fluorescence is marked by *blue triangles*, whereas the *curve* representing the YFP fluorescence is marked by *orange squares*.

chronic inflammation and autoimmune diseases (19). Therefore, a characterization of their mode of action is of considerable importance.

Because gp130 is the shared signaling receptor subunit of the IL-6-type cytokines, specificity of IL-6-RFP is a critical issue. On human cells, OSM signals through a heterodimer of gp130 and the OSM-receptor or a heterodimer of gp130 and the LIF-receptor (LIFR) (30). Even a large excess of IL-6-RFP over OSM does not inhibit OSM activity (Fig. 1, *B* and *E*), indicating that the affinity of OSM to the gp130 moiety of IL-6-RFP is very low. LIF signals through a heterodimer of gp130 and the LIFR (30). At a 60-fold molar excess of IL-6-RFP over LIF, LIF activity is moderately affected. This cross-reactivity of IL-6-RFP can be explained by low affinity binding of LIF to domains D2 and D3 of gp130 (31). However, at concentrations of IL-6-RFP sufficient to trap IL-6 in a high-affinity complex, the fusion protein is a highly specific IL-6 inhibitor. The low activity of IL-6-RFP toward OSM and LIF is in line with the ineffective neutralization of these cytokines by the full-length soluble gp130 (D1–D6). A 300-fold molar excess of sgp130 over OSM resulted in only a marginal inhibition of OSM bioactivity (7).

Interestingly, in a long term reporter gene assay that mimics IL-6 bioactivity, a short delay of IL-6-RFP application is well tolerated (Fig. 1*C*). To inhibit the early activation of STAT3 in response to IL-6, however, IL-6-RFP has to be added at least simultaneously (Fig. 1*D*). These results show that a short initial pulse of STAT3 activation is not sufficient for a robust gene induction but that the cytokine has to act for a considerable period of time. This means that, for the treatment of a disease with IL-6-RFP, the inhibitor can block the biological activity of IL-6 even when applied after the cytokine release. This is further substantiated by the results of our live cell imaging experiment. IL-6 bound to the cell surface is removed from the receptor complex upon the addition of IL-6-RFP (Fig. 5).

The shift of GFP fusion proteins in native gels can be used to detect protein-protein interactions (32). We combined the application of blue native gel electrophoresis, fluorescent fusion proteins, and advanced fluorescence gel imaging to analyze the interaction of IL-6-RFP with IL-6. YFP-IL-6 was generated by fusion of the YFP to the N terminus of IL-6. The 28 N-terminal amino acids of IL-6 are not involved in receptor binding (33). Therefore, fusion of YFP to the N terminus of IL-6 does not affect IL-6 bioactivity (26). Indeed, the interaction of YFP-IL-6 with IL-6-RFP could be visualized by a dramatic shift of the fluorescent band (Fig. 2). A 2-fold molar excess of IL-6-RFP over YFP-IL-6 is sufficient to completely trap YFP-IL-6 in a high molecular mass complex. In these fluorescence gel shift assays, the separate soluble receptor proteins sIL-6Rα and sgp130 are of lower activity, which is in

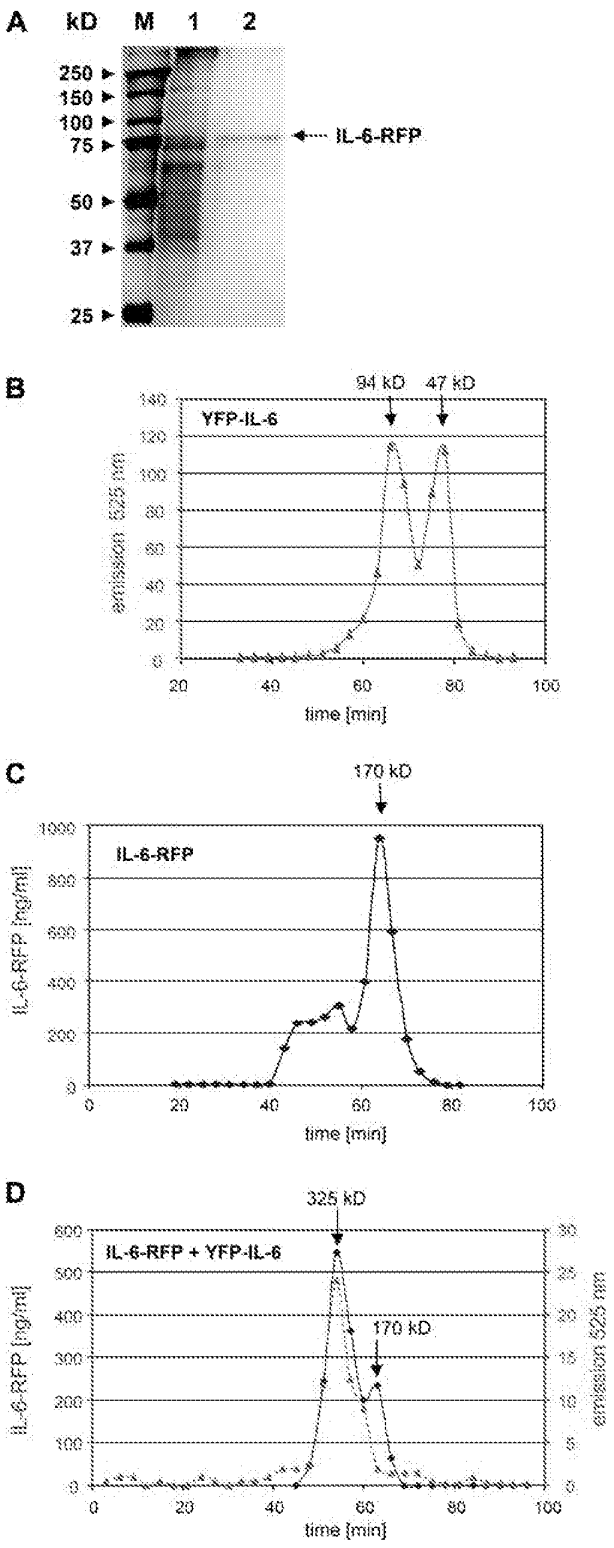


FIGURE 6. **Superdex gel filtration of YFP-IL-6 and the complex of YFP-IL-6·IL-6-RFP.** *A*, 25 μl of supernatant of baculovirus-infected insect cells containing ~25 ng of IL-6-RFP (*lane 1*) or 25 ng of purified IL-6-RFP in PBS (*lane 2*) were separated by SDS-PAGE under non-reducing conditions. Subsequently, the gel was silver-stained. *M*, marker proteins. *B*, 57 μg of YFP-IL-6 were analyzed on a calibrated Superdex 200 16/60 column (molecular mass range 10–600 kDa) at a flow rate of 1 ml/min. 3-ml fractions were collected and analyzed by fluorescence spectroscopy measuring the YFP emission at 525 nm. *C*, IL-6-RFP (17.7 μg) was analyzed by gel filtration on the same column. 3-ml fractions were analyzed by ELISA. *D*, to enable complex formation, YFP-IL-6 (5.7 μg) was incubated with IL-6-RFP in a 2-fold molar surplus (17.7 μg) for 30 min at room temperature. The complex was analyzed by gel filtration. The 3-ml fractions were analyzed by fluorescence spectroscopy to detect YFP-IL-6 and by an ELISA to determine the concentrations of IL-6-RFP. All molecular masses were calculated using a regression curve based on the separation of a known protein standard mixture.

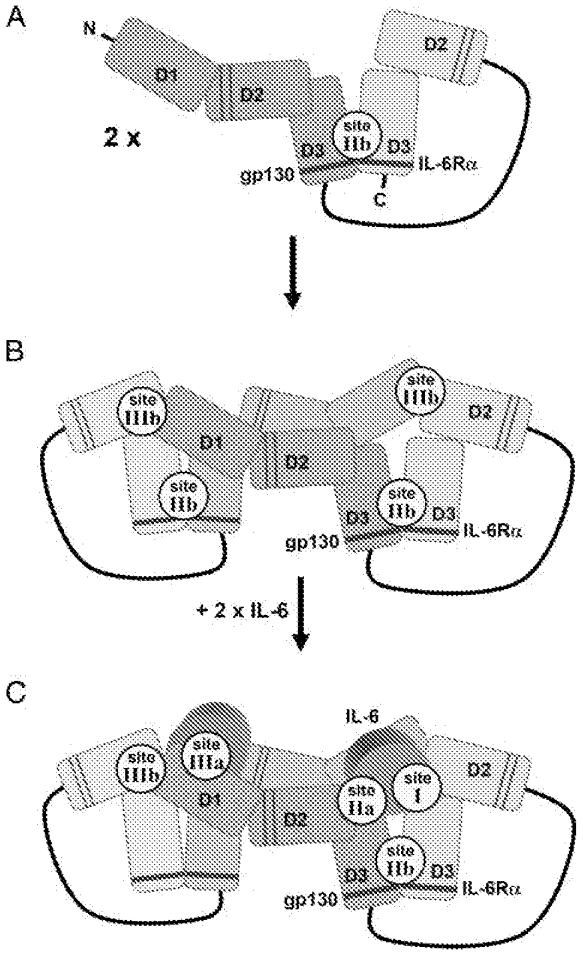


FIGURE 7. **Proposed assembly of the IL-6·IL-6-RFP complex in analogy to the hexameric IL-6 receptor complex.** Individual domains of gp130 and IL-6Rα are shown in *blue* and *green*, respectively. *Black bars* mark the conserved WSXWS motif in D3 domains, and *black lines* depict conserved disulfide bonds in D2 domains. The linker is shown as a *black line*. Interactions are designated as proposed by Boulanger *et al.* (16). Monomeric IL-6-RFP (*A*) forms a dimer (*B*) through site IIb and IIIb interactions. For clarity, in the complex of IL-6-RFP and IL-6 (*C*), only five of the ten interactions are marked. In the complex, each of the marked interactions occurs twice.

line with their lower potential to inhibit IL-6 (3). The ranking of efficiency in trapping IL-6 is IL-6-RFP > sIL-6Rα/sgp130 > sIL-6Rα > sgp130. Sgp130 alone has no detectable affinity to IL-6 (not shown). The superior activity of IL-6-RFP is also reflected in the increased stability of the YFP-IL-6·IL-6-RFP complex toward denaturation by urea, the low rate of replacement of YFP-IL-6 by non-tagged IL-6, the resistance

of the YFP-IL-6·IL-6-RFP complex toward an excess of the soluble receptor proteins (Figs. 3 and 4), and the elimination of receptor-bound YFP-IL-6 from receptor complexes at the plasma membrane by IL-6-RFP (Fig. 5).

In blue native PAGE, the mobility of the complex of YFP-IL-6 and IL-6-RFP is lower than the mobility of the albumin tetramer (260 kDa) (Fig. 2*A*). From a plot of the logarithm of molecular masses of the BSA multimers *versus* the relative migration distances (R_f not shown), we calculated an apparent molecular mass of ~290 kDa for the complex of YFP-IL-6 and IL-6-RFP. We performed gel filtration with a calibrated column (Fig. 6) to corroborate the molecular masses determined by blue native PAGE. Gel filtration confirmed the observations made by blue native PAGE. We found that preparations of YFP-IL-6 consist of a monomeric and a dimeric species (Fig. 6*B*). Dimerization of YFP-IL-6 is not caused by the YFP moiety, because YFP appears as a monomer in native PAGE (not shown). Thus, dimerization of the fusion protein is attributed to the known property of recombinant IL-6 to form dimers. The metastable IL-6 dimer has been described to dissociate in the presence of urea with a midpoint of dissociation at 3 M urea (34). The behavior of YFP-IL-6 in our gel shift assays is in agreement with the biophysical investigations on the IL-6 dimer. The mobility of the IL-6·IL-6-RFP complex does not change under conditions that lead to dissociation of the YFP-IL-6 dimer (Fig. 3), indicating that IL-6-RFP is largely complexed with monomeric YFP-IL-6. This observation is in line with the fact that IL-6 dimerizes by interactions of the gp130 binding sites of two IL-6 molecules (34). These sites are occupied upon binding of IL-6 to IL-6-RFP (Fig. 7*C*).

Unexpectedly, unliganded IL-6-RFP elutes from the gel filtration column with a retention time corresponding to a dimeric protein. Sgp130 as well as sIL-6Rα are monomeric proteins that interact in the presence of IL-6 (35). However, in the x-ray structure of the hexameric IL-6 receptor complex (16) four contacts between IL-6Rα and gp130 were identified. Possibly, in the dimeric IL-6-RFP, these contacts are partially stabilized by the covalent linkage of the corresponding IL-6Rα and gp130α domains leading to the formation of an IL-6-RFP dimer in the absence of IL-6 (Fig. 7*B*).

If we assume a preformed IL-6-RFP dimer as depicted in Fig. 7*B*, IL-6 fits well into this assembly, leading to a complex of IL-6 and IL-6-RFP built up in analogy to the hexameric IL-6 receptor complex (Fig. 7*C*). The apparent molecular masses determined by gel filtration (325 kDa) and blue native PAGE (290 kDa) are higher than the calculated molecular mass (264 kDa) of the $(IL\text{-}6)_2{\cdot}(IL\text{-}6\text{-}RFP)_2$ complex. However, the complex depicted in Fig. 7*C* is not a highly compact structure but rather extended, comprising a real cavity between the two IL-6 molecules. This architecture might explain the increased apparent molecular mass. A similar deviation between calculated and apparent molecular masses has also been observed in analysis of the soluble IL-6 receptor complex by gel filtration and analytical ultracentrifugation (35).

Taken together our analysis by blue native PAGE and gel filtration suggests that the IL-6·IL-6-RFP complex is built up in close analogy to the soluble extracellular hexameric IL-6 receptor complex. The latter complex is held together by 10 protein-protein interactions (twice the sites of I, IIa, IIb, IIIa, and IIIb) (Fig. 7*C*). The superior stability of the IL-6·IL-6-RFP complex in comparison with a similar complex built up by IL-6, sIL-6Rα, and sgp130 is caused by the stabilization of these interactions through a covalent linkage of the ligand binding domains of sIL-6Rα and sgp130. Thus, a molecular basis is provided for the high specificity and superior affinity of IL-6-RFP in comparison to other IL-6 inhibitors. Therefore, the fusion of ligand binding domains of heteromeric cytokine receptors is a promising approach for novel anti-cytokine therapies.

Acknowledgments—We thank Dr. John Wijdenes (Diaclone, Besançon, France) for the kind gift of the gp130 antibody B-T2. We thank Dr. Rene Krieg for his help with the fluorescence scanner and Andrea Küster for excellent technical assistance.

REFERENCES

1. Goldenberg, M. M. (1999) *Clin. Ther.* **21,** 75–87
2. Economides, A. N., Carpenter, L. R., Rudge, J. S., Wong, V., Koehler-Stec, E. M., Hartnett, C., Pyles, E. A., Xu, X., Daly, T. J., Young, M. R., Fandl, J. P., Lee, F., Carver, S., McNay, J., Bailey, K., Ramakanth, S., Hutabarat, R., Huang, T. T., Radziejewski, C., Yancopoulos, G. D., and Stahl, N. (2003) *Nat. Med.* **9,** 47–52
3. Ancey, C., Küster, A., Haan, S., Herrmann, A., Heinrich, P. C., and Müller-Newen, G. (2003) *J. Biol. Chem.* **278,** 16968–16972
4. Choy, E. (2003) *Ann. Rheum. Dis.* **62,** Suppl. 2, 68–69
5. Nishimoto, N., Kanakura, Y., Aozasa, K., Johkoh, T., Nakamura, M., Nakano, S., Nakano, N., Ikeda, Y., Sasaki, T., Nishioka, K., Hara, M., Taguchi, H., Kimura, Y., Kato, Y., Asaoku, H., Kumagai, S., Kodama, F., Nakahara, H., Hagihara, K., Yoshizaki, K., and Kishimoto, T. (2005) *Blood* **106,** 2627–2632
6. Heinrich, P. C., Behrmann, I., Haan, S., Hermanns, H. M., Müller-Newen, G., and Schaper, F. (2003) *Biochem. J.* **374,** 1–20
7. Narazaki, M., Yasukawa, K., Saito, T., Ohsugi, Y., Fukui, H., Koishihara, Y., Yancopoulos, G. D., Taga, T., and Kishimoto, T. (1993) *Blood* **82,** 1120–1126
8. Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T., and Kishimoto, T. (1989) *Cell* **58,** 573–581
9. Müller-Newen, G., Küster, A., Hemmann, U., Keul, R., Horsten, U., Martens, A., Graeve, L., Wijdenes, J., and Heinrich, P. C. (1998) *J. Immunol.* **161,** 6347–6355
10. Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T., and Kishimoto, T. (1990) *Cell* **63,** 1149–1157
11. Wells, J. A., and de Vos, A. M. (1996) *Annu. Rev. Biochem.* **65,** 609–634
12. Yamasaki, K., Taga, T., Hirata, Y., Yawata, H., Kawanishi, Y., Seed, B., Taniguchi, T., Hirano, T., and Kishimoto, T. (1988) *Science* **241,** 825–828
13. Simpson, R. J., Hammacher, A., Smith, D. K., Matthews, J. M., and Ward, L. D. (1997) *Protein Sci.* **6,** 929–955
14. Yawata, H., Yasukawa, K., Natsuka, S., Murakami, M., Yamasaki, K., Hibi, M., Taga, T., and Kishimoto, T. (1993) *EMBO J.* **12,** 1705–1712
15. Kurth, I., Horsten, U., Pflanz, S., Dahmen, H., Küster, A., Grötzinger, J., Heinrich, P. C., and Müller-Newen, G. (1999) *J. Immunol.* **162,** 1480–1487
16. Boulanger, M. J., Chow, D., Brevnova, E. E., and Garcia, K. C. (2003) *Science* **300,** 2101–2104
17. Bravo, J., and Heath, J. K. (2000) *EMBO J.* **19,** 2399–2411
18. Müller-Newen, G. (2003) *Science's STKE* **2003,** pe40
19. Dinarello, C. A. (2003) *Nat. Med.* **9,** 20–22
20. Arcone, R., Pucci, P., Zappacosta, F., Fontaine, V., Malorni, A., Marino, G., and Ciliberto, G. (1991) *Eur. J. Biochem.* **198,** 541–547
21. Aarden, L. A., De Groot, E. R., Schaap, O. L., and Lansdorp, P. M. (1987) *Eur. J. Immunol.* **17,** 1411–1416
22. Weiergräber, O., Hemmann, U., Küster, A., Müller-Newen, G., Schneider,

J., Rose-John, S., Kurschat, P., Brakenhoff, J. P., Hart, M. H., Stabel, S., and Heinrich, P. C. (1995) *Eur. J. Biochem.* **234,** 661–669
23. Helle, M., Boeije, L., de Groot, E., de Vos, A., and Aarden, L. (1991) *J. Immunol. Methods* **138,** 47–56
24. Schagger, H., and von Jagow, G. (1991) *Anal. Biochem.* **199,** 223–231
25. Sadtler, S., Laube, B., Lashub, A., Nicke, A., Betz, H., and Schmalzing, G. (2003) *J. Biol. Chem.* **278,** 16782–16790
26. Giese, B., Roderburg, C., Sommerauer, M., Wortmann, S. B., Metz, S., Heinrich, P. C., and Müller-Newen, G. (2005) *J. Cell Sci.* **118,** 5129–5140
27. Heinrich, P. C., Castell, J. V., and Andus, T. (1990) *Biochem. J.* **265,** 621–636
28. Honda, M., Yamamoto, S., Cheng, M., Yasukawa, K., Suzuki, H., Saito, T., Usugi, Y., Tokunaga, T., and Kishimoto, T. (1992) *J. Immunol.* **148,** 2175–2180
29. Müller-Newen, G., Köhne, C., Keul, R., Hemmann, U., Müller-Esterl, W., Wijdenes, J., Brakenhoff, J. P., Hart, M. H., and Heinrich, P. C. (1996) *Eur. J. Biochem.* **236,** 837–842
30. Gearing, D. P., Comeau, M. R., Friend, D. J., Gimpel, S. D., Thut, C. J., McGourty, J., Brasher, K. K., King, J. A., Gillis, S., Mosley, B., Ziegler, S. F., and Cosman, D. (1992) *Science* **255,** 1434–1437
31. Boulanger, M. J., Bankovich, A. J., Kortemme, T., Baker, D., and Garcia, K. C. (2003) *Mol. Cell* **12,** 577–589
32. Kiessig, S., Reissmann, J., Rascher, C., Kullertz, G., Fischer, A., and Thunecke, F. (2001) *Electrophoresis* **22,** 1428–1435
33. Brakenhoff, J. P., Hart, M., and Aarden, L. A. (1989) *J. Immunol.* **143,** 1175–1182
34. Matthews, J. M., Hammacher, A., Howlett, G. J., and Simpson, R. J. (1998) *Biochemistry* **37,** 10671–10680
35. Ward, L. D., Howlett, G. J., Discolo, G., Yasukawa, K., Hamacher, A., Moritz, R. L., and Simpson, R. J. (1994) *J. Biol. Chem.* **269,** 23286–23289

Characterization of the Interleukin (IL)-6 Inhibitor IL-6-RFP: FUSED RECEPTOR DOMAINS ACT AS HIGH AFFINITY CYTOKINE-BINDING PROTEINS

Silke Metz, Monique Wiesinger, Michael Vogt, Heike Lauks, Günther Schmalzing, Peter C. Heinrich and Gerhard Müller-Newen

J. Biol. Chem. 2007, 282:1238-1248.
doi: 10.1074/jbc.M606885200 originally published online November 3, 2006

Espacenet

Bibliographic data: KR20110047179 (A) — 2011-05-06

New oxazolidin and indole compounds, process for the preparation thereof and pharmaceutical composition comprising the same

Inventor(s):

Applicant(s):

Classification: **- international:** ***A61K31/404; A61P29/00; C07D209/26***
- cooperative:

Application number: KR20110035774 20110418 Global Dossier

Priority number(s): KR20110035774 20110418

Also published as: KR101133769 (B1)

Abstract of KR20110047179 (A)

PURPOSE: A pharmaceutical composition containing novel oxazolidine and indole compound, or pharmaceutically acceptable salt is provided to suppress IL-6 or IL-11 signal transduction system and to be used as an anticancer agent and anti-inflammatory agent. CONSTITUTION: An indole compound is denoted by chemical formula 1. A pharmaceutical composition for preventing and treating inflammatory diseases or cancer contains 0.0001-10 weight% of indole compound of chemical formula 1 or pharmaceutically acceptable salt. The composition is manufactured in the formulation of powders, granules, tablets, capsules, suspensions, emulsions, syrups, suppository, and excipient. The pharmaceutical composition is administered by oral, rectal or venous, muscular, subcutaneous, or intracerebroventricular injection.

(19) 대한민국특허청(KR)
(12) 공개특허공보(A)

(11) 공개번호 10-2011-0047179
(43) 공개일자 2011년05월06일

(51) Int. Cl.
C07D 209/26 (2006.01) *A61K 31/404* (2006.01)
A61P 29/00 (2006.01)
(21) 출원번호 10-2011-0035774(분할)
(22) 출원일자 2011년04월18일
심사청구일자 2011년04월18일
(62) 원출원 특허 10-2009-0006136
원출원일자 2009년01월23일
심사청구일자 2009년01월23일

(71) 출원인
한국생명공학연구원
대전 유성구 어은동 52번지
(72) 발명자
노문철
대전광역시 유성구 전민동 청구아파트 104동 1605호
김영국
대전시 유성구 전민동 엑스포아파트 409동 1601호
(뒷면에 계속)
(74) 대리인
손민

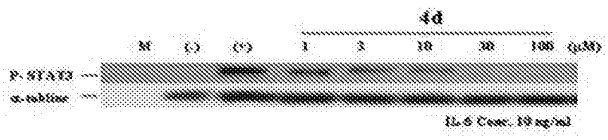
전체 청구항 수 : 총 7 항

(54) 신규한 옥사졸리딘계 및 인돌계 화합물, 이의 제조방법 및 이를 포함하는 약학 조성물

(57) 요 약

본 발명은 신규한 옥사졸리딘계 및 인돌계 화합물, 또는 이의 약학적으로 허용가능한 염, 이의 제조방법, 및 이를 포함하는 약학 조성물에 관한 것이다. 본 발명의 신규 화합물은 염증성 질환 및 암을 유발하는 신호전달과정에서 중요한 매개자로 작용하는 IL-6 또는 IL-11 신호전달 체계를 저해하는 활성이 우수하므로, 항암제 및 항염증제로 유용하게 사용될 수 있다.

대 표 도 - 도3



(72) 발명자

최용석

서울시 동작구 신대방1동 동작상떼빌 103동 603호

이경

대전광역시 서구 둔산동 1369 넥서스밸리 B동 313호

최정호

대전광역시 중구 오류동 151-8 크럭스빌 402호

이우송

전라북도 정읍시 신정동 1404번지 한국생명공학연구원 전북분원 생물산업 기술 연구센터

이승웅

대전광역시 서구 괴정동 한신아파트 104동 105호

박수진

광주광역시 서구 상무1동 모아제일아파트 103동 1701호

장종선

대구광역시 동구 용계동 397-9번지

정화영

경상북도 상주시 외서면 봉강1리 290

이 발명을 지원한 국가연구개발사업

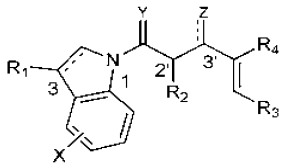
과제고유번호	KGM2410912
부처명	교육과학기술부
연구관리전문기관	기초기술연구회
연구사업명	기관주요사업
연구과제명	감염 염증 예방 및 치료용 친환경 활성 생물소재 개발
기여율	1/1
주관기관	한국생명공학연구원
연구기간	2009.01.01 ~ 2009.12.31

특허청구의 범위

청구항 1

하기 화학식 1로 표시되는 인돌계 화합물 또는 이의 약학적으로 허용되는 염:

<화학식 1>



상기에서 R_1은 수소, 또는 치환 또는 비치환된 C_{1-10} 알킬이고;

R_2는 수소, 또는 C_{1-10}의 직쇄, 측쇄 또는 사이클로 알킬이고;

R_3는 수소, 또는 C_{1-10}의 직쇄, 측쇄 또는 사이클로 알킬, 또는 치환 또는 비치환된 벤질이고;

R_4는 수소, 또는 C_{1-10}의 직쇄, 측쇄 또는 사이클로 알킬, 또는 치환 또는 비치환된 벤질이고;

X는 수소, 할로겐, 하이드록시, 메톡시, 치환 또는 비치환된 C_{1-10} 알킬이고;

Y는 수소 또는 산소이고;

Z는 하이드록시, C_{1-10}의 알콕시, $-OCOCH_3$ 또는 산소이고; 및

상기 ------ 는 단일 또는 이중 결합이다.

청구항 2

제1항에 있어서, 상기 R_1은 하이드록시에틸 또는 터트-부틸디메틸실릴옥시에틸이고;

R_2는 C_{1-5}의 직쇄, 측쇄 또는 사이클로 알킬이고;

R_3는 수소이고;

R_4는 수소 또는 C_{1-5}의 직쇄, 측쇄 또는 사이클로 알킬이고;

X는 수소, 할로겐, 하이드록시, 메톡시, 치환 또는 비치환된 알킬이고;

Y는 수소 또는 산소이고;

Z는 하이드록시, C_{1-5}의 알콕시, $-OCOCH_3$ 또는 산소이고; 및

상기 ------ 는 단일 또는 이중 결합인 화합물, 또는 이의 약학적으로 허용가능한 염.

청구항 3

제1항에 있어서, 상기 화합물은

1)
(2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-3-하이드록시-2-메틸-4-메틸렌노난-1-온,

2) (2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-2-메틸-4-메틸렌-1-옥소노난-3-일 아세테이트,

3) (2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌-1-옥소노난-3-일 아

세테이트,

4) (2S,3S)-1-(3-(2-하이드록시에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌-1-옥소노난-3-일 아세테이트,

5) (2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)-1H-인돌-1-일)-3-하이드록시-2-메틸-4-메틸렌노난-1-온,

6) (2S,3S)-3-하이드록시-1-(3-(2-하이드록시에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌노난-1-온,

7) (2R,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-2-메틸-4-메틸렌노난-3-올,

8) (2R,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌노난-3-올,

9) (2R,3S)-1-(3-(2-하이드록시에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌노난-3-올,

10) (2S,3S)-3-하이드록시-1-(3-(2-하이드록시에틸)인돌린-1-일)-2-메틸-4-메틸렌노난-1-온,

11) (2S,3S)-1-(3-(2-하이드록시에틸)인돌린-1-일)-2-메틸-4-메틸렌-1-옥소노난-3-일 아세테이트 및

12) (2R,3S)-1-(3-(2-하이드록시에틸)인돌린-1-일)-2-메틸-4-메틸렌노난-3-올

으로 이루어진 군으로부터 선택되는 화합물 또는 이의 약학적으로 허용가능한 염.

청구항 4

제1항 내지 제3항 중 어느 한 항의 화합물 또는 이의 약학적으로 허용가능한 염을 포함하는, 염증성 질환 또는 암의 예방 및 치료용 약학 조성물.

청구항 5

제4항에 있어서, 상기 염증성 질환 또는 암은 IL-6 또는 IL-11 에 의해 유발되는 것인 조성물.

청구항 6

제4항에 있어서, 상기 염증성질환은 류마티스 관절염, 골다공증, 형질구 증가증, 초면역 글로불린 혈증, 빈혈, 신염, 악액질, 맥관증식신염, 다발성 경화증, 포도막염, 만성 갑상선염, 지연과민증, 접촉피부염 아토피성 피부염, 전신성 홍반증, 크론병, 췌장염, 건선, 연소성 특발성 위축증, 당뇨병 및 알쯔하이머로 구성되는 군으로부터 선택되는 질환인 조성물.

청구항 7

제4항에 있어서, 상기 암은 췌장암, 유방암, 전립선암, 뇌종양, 두경부암종, 흑색종, 골수종, 흑색종, 백혈병, 림프종, 간암, 위암, 결장암, 골암, 자궁암, 난소암, 직장암, 식도암, 소장암, 항문부근암, 결장암, 나팔관암종, 자궁내막암종, 자궁경부암종, 질암종, 음문암종, 호지킨병, 방광암, 신장암, 수뇨관암, 신장세포암종, 신장골반암종 및 중추신경계 종양로 구성되는 군으로부터 선택되는 암인 조성물.

명 세 서

기 술 분 야

[0001] 본 발명은 신규 합성 화합물인 옥사졸리딘계 및 인돌계 화합물들, 이들의 합성방법 또는 이들의 약학적으로 허용가능한 염을 유효성분으로 함유하는 항염증 및 항암제에 관한 것이다.

배 경 기 술

[0002] 인터류킨-6 (IL-6) 는 B 세포 자극 인자 2 (BSF2) 또는 인터페론β2 (INF-β2)로도 불리는 사이토카인이다. IL-6는 B 임파구의 활성화에 관여하는 분화인자로서 발견되었다 (Hirano, T. et al., Nature (1986) 324, 73-76). 그 후, 여러 가지 세포의 기능에 영향을 미치는 다기능 사이토카인이라는 것이 밝혀졌다 (Akira, S. et al., Adv. in Immunology (1993) 54, 1-78). IL-6는 세포막위에 두 종류의 단백질을 매개로 그 생물학적 활성을 전달한다. 하나는 IL-6가 결합하는 단백질인 IL-6 수용체이다. IL-6 수용체는 세포막을 관통하여 발현되어있는 분자량 약 80kD의 막결합형 단백질이다. 다른 하나는, 비리간드 결합성의 시그날 전달에 속하는 분자량 약 130kD의 막단백질 gp130 이다. IL-6와 IL-6 수용체는 IL-6/IL-6 수용체 복합체를 형성하고, 이어서 gp130과

결합한다. (Taga et al., J. Exp. Med. (1987) 166, 967). 리간드와 수용체들의 결합 후, 세포내에서는 Janus Kinases 2 (JAK2)가 인산전이반응(transphosphorylation)에 의해 활성화된다. 활성화된 JAK2에 의해 수용체 세포질 도메인(cytoplasmic domains)의 여러 타이로신 잔기(tyrosine residues)가 인산화 (phosphorylation)되고 이것은 SH2나 다른 인산타이로신 결합 모티프(phosphotyrosine binding motif)를 가지고 있는 STAT3 (signal transducers and activators of transcription 3)와 같은 세포질(cytoplasm) 내 단백질의 docking site 역할을 하게 된다. 수용체의 세포질 도메인(cytoplasmic domain)에 결합한 STAT3는 JAK2에 의해 인산화 (phosphorylation)가 된 후 수용체에서 떨어져 나온다. 활성화된 STAT3들은 세포질내에 서로서로 결합하여 호모(homo-) 또는 헤테로다이머(heterodimer)를 이룬 후 핵(nucleus) 내로 들어가 목적 유전자의 인식 서열(recognition sequence)에 결합하여 전사(transcription)을 증가시킨다 (Levy, D.E., 등, Nat Rev Mol Cell Biol, 2002, 3, 651-62, Darnell, J.E., J.r., Science, 1997, 277, 1630-1635).

[0003] 이러한 IL-6에 의해 유도되는 신호전달체계는 염증성 질환 및 여러 암 질환과의 관련이 보고되어 있으며, 따라서 IL-6에 의해 유도되는 신호전달체계의 억제는 치료적으로 유용하다. 현재, IL-6의 신호전달체계의 억제하는 기능에 대한 연구는 항 IL-6 R 항체가 가장 많이 연구되어 있다. 이 항 IL-6 R 항체는 류마티스성 관절염에 대하여 활액 세포 성장 억제제가 보고되었고 (국제특허공개 제98/11020호), 형질구 증가증, 초면역 글로불린 혈증, 빈혈, 신염, 악액질, 류마티스성 관절염, 목축업자 질병, 및 맥관증식신염과 같은 IL-6 산물에 기여하는 질병의 치료에 기술된 바 있다 (국제특허공개 제96/12503호). 다발성 경화증, 포도막염, 만성 갑상선염, 지연과민증, 접촉피부염 및 아토피성 피부염과 같은 민감성 T 세포 관련 질병의 예방/보호제에서도 기술되어있고 (국제특허공개 제98/42377호), 전신성 홍반증의 치료제를 기술하는 특허도 보고 있다 (국제특허공개 제98/42377호). 또한, 크론병의 치료제를 기술하는 보고에서도 (국제특허공개 제99/47170], 이것의 활성성분은 항-IL-6R 항체이었다. 췌장염의 치료제를 기술하고 있는 특허도 활성성분으로 보고되었고 (국제특허공개 제00/10607), 건선의 치료제를 기술하고 있는 특허인 국제특허공개 제02/3492호에서도 활성성분은 항-IL-6R 항체이다. 추가로, 연소성 특발성 위축증의 치료제를 기술하고있는 국제특허공개공보 제02/080969호에서도 이것의 활성성분은 항-IL-6R 항체이다. 그러나, 이들 단백질은 외래 단백질로서 인지되어질 수 있는 에피토프를 가질 수 있으며 치료제로서 사용될 경우 여전히 면역원성일 수 있다. 그러나 단백질이 아닌 작은 분자 화합물(small molecule compound)들은 이러한 면역체계에 인지되지 않아 현재 많은 연구가 이루어지고 있다.

[0004] 또한 암과 관련되어 IL-6 신호전달체계는 그 중간 매개인자인 STAT3와 많은 관련이 있다. 이것은 골수종, 유방암종, 전립선 암, 뇌 종양, 두경부 암종, 흑색종, 백혈병 및 림프종, 특히 만성 골수성 백혈병 및 다발성 골수종을 포함하여 여러 형태의 암에 관여하는 것으로 보고되었다 (Niu, 등, Cancer Res., 1999, 59, 5059-5063). 쥐 및 인간 전립선 암 양쪽에서 유래된 세포들은 구조적으로 활성화된 STAT3을 갖는 것으로 밝혀졌으며, STAT3는 일부 급성 백혈병(Gouilleux-Gruart, V. 등, Leuk.Lymphoma, 1997, 28, 83-88) 및 T 세포 림프종 (Yu,C.L. 등, J.Immunol., 1997, 159, 5206-5210) 에서 구조적으로 활성화되는 것으로 밝혀졌다. 흥미롭게도, STAT3은 만성 림프성 백혈병에서 세린 잔기 위에 구조적으로 인산화 되는 것으로 밝혀졌다 (Frank, D.A., 등, J.Clin.Invest., 1997, 100, 3140-3148). STAT3은, 다발성 골수종을 가진 환자로부터의 골수 단핵 세포 및 배양액 양쪽 모두에서, 골수종 종양 세포에서 구조적으로 활성인 것으로 밝혀졌다. 이러한 세포는 Fas-매개 세포 고사에 내성이고 높은 수준의 Bcl-xL을 발현한다. STAT3 시그날링은 세포고사에 대한 내성을 부여함으로써 골수종 종양 세포의 생존을 위해 필수적인 것으로 밝혀졌다 (Catlett-Falcone, R. 등, Immunity, 1999, 10, 105-115). 한편, 최근에는 췌장암을 비롯한 Ras 에 의해 유도되는 암 환자군에서 이상적으로 IL-6가 분비되고, IL-6를 제거함으로서 Ras에 의한 종양세포의 성장과 혈관생성이 억제될 뿐 아니라 종양의 크기가 감소됨이 보고되었다 (Brooke Ancrile 등, Gene & Development, 2007, 21, 1714-1719). 또한 EGFR이 변이된 폐 선암(lung adenocarcinoma)에서 IL-6가 과발현됨으로서 STAT3가 활성화됨이 밝혀지면서 IL-6에 의한 gp130/JAK/STAT3 경로가 항암치료에 있어서 새로운 타켓으로 부각되고 있다 (Sizhi Paul Gao 등, J. Clin. Invest. 2007, 117, 38463856).

[0005] 한편, 인터류킨-11 (IL-11)은 IL-6 family에 속해 있는 염증성 사이토카인으로 IL-6와 거의 같은 신호전달체계를 갖는 것으로 조혈, 면역반응, 염증 및 각종 암세포에서 그 발현이 증가되어 암 진행에 중요한 역할을 하는 것으로 알려지고 있다 (Jackson CB 등 J Pathol 2007, 213, 140-151). 최근 IL-11이 그 수용체인 IL-11Rα와 gp130에 결합하여 위암, 대장암 세포 증식 및 암 침윤을 촉진시키는 작용이 있음이 보고되었고 (Nakayama T 등 Int J Oncol, 2007, 30, 825-833, Yoshizaki A 등, Int J Oncol 2006, 29, 869-876), Ernst 등은 IL-11/Stat3 신호에 의해 smad7이 활성화되고 동시에 TGF β 시그날을 유도하는 smad activator가 차단됨으로서 oncogenic

program (antiapoptotic gene, proangiogenic gene, proliferative gene)이 활성화되어 염증관련 위종양이 유도된다고 보고하였다(Ernst 등 J. Clin. Invest 2008, 118(5), 1728-1738). 따라서 IL-11에 의한 gp130/JAK/STAT3 경로가 항암치료에 있어서 새로운 타켓으로 부각되고 있다.

[0006] 이에, 본 발명자들은 유기합성 시료로 부터 IL-6 및 IL-11에 의해 유도되는 신호전달체계의 저해제를 탐색한 결과, 신규 합성한 옥사졸리딘계 및 인돌계 화합물들이 IL-6 및 IL-11에 의해 유도되는 신호전달체계를 저해하는 활성이 우수하므로 염증성 질환 및 암 치료제로 유용하다는 것을 규명함으로써 본 발명을 완성하였다.

발명의 내용

해결하려는 과제

[0007] 본 발명의 하나의 목적은 신규한 옥사졸리딘계, 인돌계 화합물, 또는 이의 약학적으로 허용가능한 염을 제공하는 것이다.

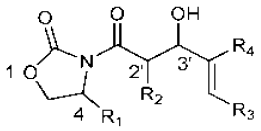
[0008] 본 발명의 또 하나의 목적은 상기 신규 화합물 또는 이의 약학적으로 허용가능한 염을 포함하는, 염증성 질환 또는 암의 예방 및 치료용 약학 조성물을 제공하는 것이다.

과제의 해결 수단

[0009] 이하, 본 발명을 상세히 설명한다.

[0010] 하나의 양태로서, 본 발명은 하기 화학식 1로 표시되는 옥사졸리딘계 화합물 또는 이의 약학적으로 허용되는 염에 관한 것이다.

[0011] <화학식 1>

[0012] 

[0013] 상기에서 R_1은 수소 또는 C_{1-10}의 직쇄, 측쇄 또는 사이클로 알킬, 또는 치환 또는 비치환된 벤질이고; R_2는 수소, 또는 C_{1-10}의 직쇄, 측쇄 또는 사이클로 알킬이고; R_3는 수소, 또는 C_{1-10}의 직쇄, 측쇄 또는 사이클로 알킬, 또는 치환 또는 비치환된 벤질이고; R_4는 수소, 또는 C_{1-10}의 직쇄, 측쇄 또는 사이클로 알킬, 또는 치환 또는 비치환된 벤질이거나, 또는 R_3 및 R_4가 함께 사이클로헥센을 형성한다.

[0014] 보다 바람직하게는, 상기 R_1은 아이소프로필 또는 벤질이고; R_2는 C_{1-5}의 직쇄, 측쇄 또는 사이클로 알킬이고; R_3는 수소이고; 및 R_4는 C_1, C_{4-7}알킬, 아이소프로필, 또는 벤질이거나, 또는 R_3 및 R_4가 함께 사이클로헥센을 형성한다.

[0015] 본 발명의 화학식 1의 화합물 중 바람직한 화합물은 구체적으로 하기와 같다:

[0016] 1) (S)-3-((2S,3S)-3-하이드록시-2,4-디메틸펜트-4-에노일)-4-이소프로필옥사졸리딘-2-온(4a),

[0017] 2) (S)-3-((2S,3S)-3-하이드록시-2,5-디메틸-4-메틸렌헥사노일)-4-이소프로필옥사졸리딘-2-온(4b),

[0018] 3) (S)-3-((2S,3S)-3-하이드록시-2-메틸-4-메틸렌옥타노일)-4-이소프로필옥사졸리딘-2-온(4c),

[0019] 4) (S)-3-((2S,3S)-3-하이드록시-2-메틸-4-메틸렌노나노일)-4-이소프로필옥사졸리딘-2-온(4d),

[0020] 5) (S)-3-((2S,3S)-3-하이드록시-2-메틸-4-메틸렌데카노일)-4-이소프로필옥사졸리딘-2-온(4e),

[0021] 6) (S)-3-((2S,3S)-3-하이드록시-2-메틸-4-메틸렌운데카노일)-4-이소프로필옥사졸리딘-2-온(4f),

[0022] 7) (S)-3-((2S,3S)-4-벤질-3-하이드록시-2-메틸펜트-4-에노일)-4-이소프로필옥사졸리딘-2-온(4g),

[0023] 8) (S)-3-((2S,3S)-3-사이클로헥세닐-3-하이드록시-2-메틸프로파노일)-4-이소프로필옥사졸리딘-2-온(4h),

[0024] 9) (R)-3-((2R,3R)-3-하이드록시-2-메틸-4-메틸렌노나노일)-4-이소프로필옥사졸리딘-2-온(5),

[0025] 10) (S)-4-벤질-3-((2S,3S)-3-하이드록시-2-메틸-4-메틸렌노나노일)옥사졸리딘-2-온(7a),

[0026] 11) (S)-4-벤질-3-((2R,3S)-3-하이드록시-2-메틸-4-메틸렌노나노일)옥사졸리딘-2-온(7b), 및

[0027] 12) (S)-3-((2S,3R)-3-하이드록시-2-메틸노나노일)-4-이소프로필옥사졸리딘-2-온(9).

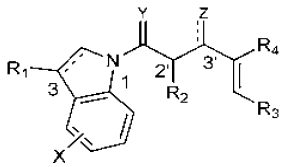
[0028] 표 1

화합물	R1	R4	4	2'	3'
4a	$CH(CH_3)_2$	CH_3	S	S	S
4b	$CH(CH_3)_2$	$CH(CH_3)_2$	S	S	S
4c	$CH(CH_3)_2$	C_4H_9	S	S	S
4d	$CH(CH_3)_2$	C_5H_{11}	S	S	S
4e	$CH(CH_3)_2$	C_6H_{13}	S	S	S
4f	$CH(CH_3)_2$	C_7H_{15}	S	S	S
4g	$CH(CH_3)_2$	benzyl	S	S	S
4h	$CH(CH_3)_2$	1-Cyclohexene	S	S	S
5	$CH(CH_3)_2$	$CH(CH_3)_2$	R	R	R
7a	benzyl	C_5H_{11}	S	S	S
7b	benzyl	C_5H_{11}	S	R	S
9	$CH(CH_3)_2$	C_6H_{15}	S	S	R

[0029] 본 발명의 화학식 1의 옥사졸리딘계 화합물은 약학적으로 허용가능한 염의 형태로 사용할 수 있으며, 통상의 방법에 의해 제조되는 모든 염, 수화물 및 용매화물이 포함된다. 염으로는 약학적으로 허용가능한 유리산(free acid)에 의해 형성된 산부가염이 유용하다. 유리산으로는 무기산과 유기산을 사용할 수 있으며, 무기산으로는 염산, 브롬산, 황산, 인산 등을 사용할 수 있고, 유기산으로는 구연산, 초산, 젖산, 주석산, 푸마르산, 포름산, 프로피온산, 옥살산, 트리플루오로아세트산, 메탄술폰산, 벤젠술폰산, 말레인산, 벤조산, 글루콘산, 글리콜산, 숙신산, 4-모폴린에탄술폰산, 캠포술폰산, 4-니트로벤젠술폰산, 히드록시-O-술폰산, 4-톨루엔술폰산, 칼룩투론산, 엠보산, 글루탐산, 아스파르트산 등을 사용할 수 있다.

[0030] 또 하나의 양태로서, 본 발명은 하기 화학식 2로 표시되는 인돌계 화합물 또는 이의 약학적으로 허용되는 염에 관한 것이다.

[0031] <화학식 2>

[0032] 

[0033] 상기에서 R_1은 수소, 또는 치환 또는 비치환된 C_{1-10} 알킬이고; R_2는 수소, 또는 C_{1-10}의 직쇄, 측쇄 또는 사이클로 알킬이고; R_3는 수소, 또는 C_{1-10}의 직쇄, 측쇄 또는 사이클로 알킬, 또는 치환 또는 비치환된 벤질이고; R_4는 수소, 또는 C_{1-10}의 직쇄, 측쇄 또는 사이클로 알킬, 또는 치환 또는 비치환된 벤질이고; X는 수소, 할로겐, 하이드록시, 메톡시, 치환 또는 비치환된 C_{1-10} 알킬이고; Y는 수소 또는 산소 이고; Z는 하이드록시, C_{1-10}의 알콕시,

$-OCOCH_3$ 또는 산소이고; 상기 ------ 는 단일 또는 이중 결합이다.

[0034] 보다 바람직하게는, R_1은 하이드록시에틸 또는 터트-부틸디메틸실릴옥시에틸이고; R_2는 C_{1-5}의 직쇄, 측쇄 또는 사이클로 알킬이고; R_3는 수소이고; R_4는 수소 또는 C_{1-5}의 직쇄, 측쇄 또는 사이클로 알킬이고; X는 수소, 할로겐, 하이드록시, 메톡시, 치환 또는 비치환된 알킬이고; Y는 수소 또는 산소이고; Z는 하이드록시, C_{1-5}의 알콕시, $-OCOCH_3$ 또는 산소이고; 및 상기 ------ 는 단일 또는 이중 결합이다.

[0035] 본 발명의 화학식 2의 화합물 중 바람직한 화합물은 구체적으로 하기와 같다:

[0036] 1) (2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-3-하이드록시-2-메틸-4-메틸렌노난-1-온(13a),

[0037] 2) (2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-2-메틸-4-메틸렌-1-옥소노난-3-일 아세테이트(14a),

[0038] 3) (2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌-1-옥소노난-3-일 아세테이트(15a),

[0039] 4) (2S,3S)-1-(3-(2-하이드록시에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌-1-옥소노난-3-일 아세테이트(15b),

[0040] 5) (2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)-1H-인돌-1-일)-3-하이드록시-2-메틸-4-메틸렌노난-1-온(16a),

[0041] 6) (2S,3S)-3-하이드록시-1-(3-(2-하이드록시에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌노난-1-온(16b),

[0042] 7) (2R,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-2-메틸-4-메틸렌노난-3-올(17a),

[0043] 8) (2R,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌노난-3-올(18a),

[0044] 9) (2R,3S)-1-(3-(2-하이드록시에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌노난-3-올(18b),

[0045] 10) (2S,3S)-3-하이드록시-1-(3-(2-하이드록시에틸)인돌린-1-일)-2-메틸-4-메틸렌노난-1-온(13b),

[0046] 11) (2S,3S)-1-(3-(2-하이드록시에틸)인돌린-1-일)-2-메틸-4-메틸렌-1-옥소노난-3-일 아세테이트(14b) 및

[0047] 12) (2R,3S)-1-(3-(2-하이드록시에틸)인돌린-1-일)-2-메틸-4-메틸렌노난-3-올(17b).

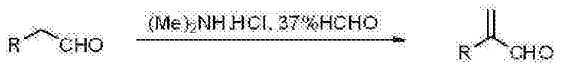
[0048] 표 2

화합물	R1	Y	Z	인돌유도체결합
13a	$(CH_2)_2$-OTBS	O	H	단일결합
14a	$(CH_2)_2$-OTBS	O	$OCOCH_3$	단일결합
15a	$(CH_2)_2$-OTBS	O	$OCOCH_3$	이중결합
15b	$(CH_2)_2$-OH	O	$OCOCH_3$	이중결합
16a	$(CH_2)_2$-OTBS	O	H	이중결합
16b	$(CH_2)_2$-OH	O	H	이중결합
17a	$(CH_2)_2$-OTBS	H	H	단일결합
18a	$(CH_2)_2$-OTBS	H	H	이중결합
18b	$(CH_2)_2$-OH	H	H	이중결합
13b	$(CH_2)_2$-OH	O	H	단일결합
14b	$(CH_2)_2$-OH	O	$OCOCH_3$	단일결합
17b	$(CH_2)_2$-OH	H	H	단일결합

[0049] 본 발명의 화학식 2의 인돌계 화합물은 약학적으로 허용가능한 염의 형태로 사용할 수 있으며, 통상의 방법에 의해 제조되는 모든 염, 수화물 및 용매화물이 포함된다. 염으로는 약학적으로 허용가능한 유리산(free acid)에

의해 형성된 산부가염이 유용하다. 유리산으로는 무기산과 유기산을 사용할 수 있으며, 무기산으로는 염산, 브롬산, 황산, 인산 등을 사용할 수 있고, 유기산으로는 구연산, 초산, 젖산, 주석산, 푸마르산, 포름산, 프로피온산, 옥살산, 트리플루오로아세트산, 메탄술폰산, 벤젠술폰산, 말레인산, 벤조산, 글루콘산, 글리콜산, 숙신산, 4-모폴린에탄술폰산, 캠포술폰산, 4-니트로벤젠술폰산, 히드록시-0-술폰산, 4-톨루엔술폰산, 칼룩투론산, 엠보산, 글루탐산, 아스파르트산 등을 사용할 수 있다.

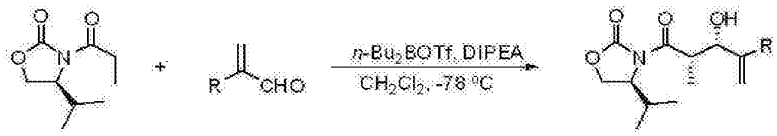
[0050] <반응식 1>



2b (R=$CH(CH_3)_2$)
2c (R=C_4H_9)
2d (R=C_5H_{11})
2e (R=C_6H_{13})
2f (R=C_7H_{15})
2g (R=Bn)

[0051]

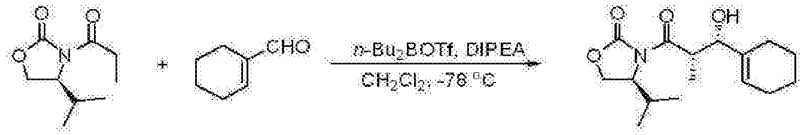
[0052] <반응식 2>



3a (S-이성질체)	**2a** (R=CH_3)	**4a** (R = CH3)
	2b (R=$CH(CH_3)_2$)	**4b** (R=$CH(CH_3)_2$)
	2c (R=C_4H_9)	**4c** (R=C_4H_9)
	2d (R=C_5H_{11})	**4d** (R=C_5H_{11})
	2e (R=C_6H_{13})	**4e** (R=C_6H_{13})
	2f (R=C_7H_{15})	**4f** (R=C_7H_{15})
	2g (R=Bn)	**4g** (R=Bn)

[0053]

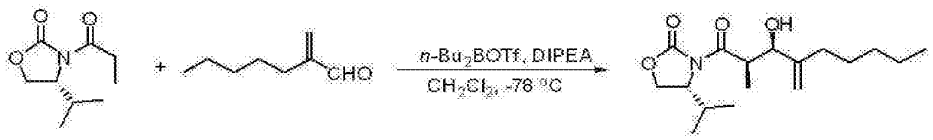
[0054] <반응식 3>



3a 2h 4h

[0055]

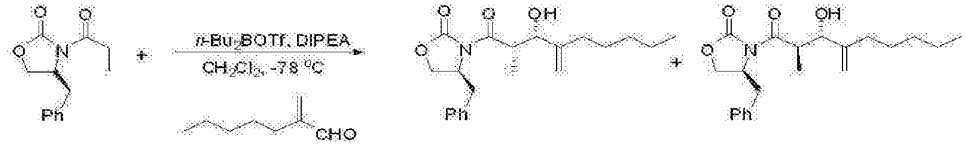
[0056] <반응식 4>



3b (R-이성질체) 2d 5

[0057]

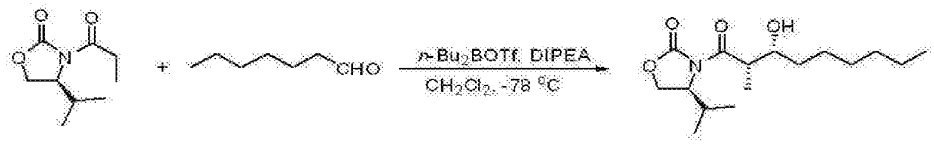
[0058] <반응식 5>



6 7a (9 : 1) 7b

[0059]

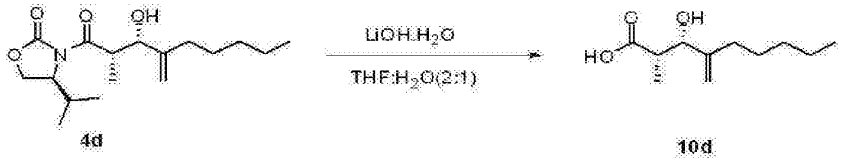
[0060] <반응식 6>



3a 8 9

[0061]

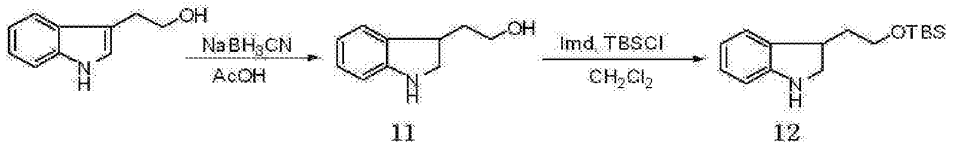
[0062] <반응식 7>



4d 10d

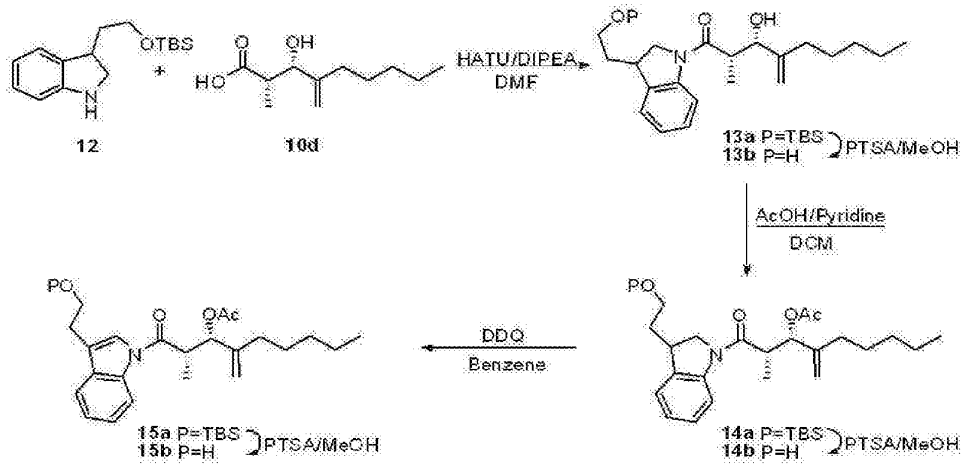
[0063]

[0064] <반응식 8>



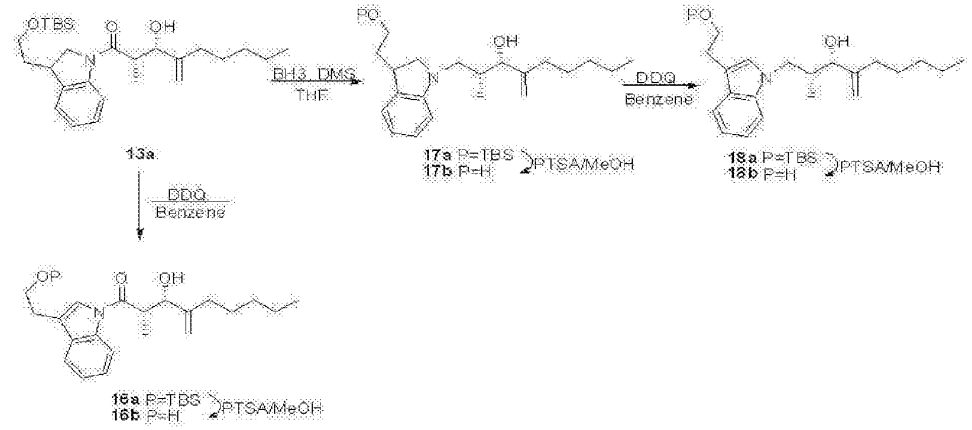
[0065]

[0066] <반응식 9>



[0067]

[0068] <반응식 10>



[0069]

[0070] 상기 각 반응 또는 모든 반응이 완결된 후의 생성물은 통상적인 후처리 방법, 예를 들면 크로마토그래피, 재결정화 등의 방법에 의해 분리 및 정제할 수 있다.

[0071] 본 발명의 아이소프로필옥사졸리딘계 화합물인 화학식 1과 인돌계 화합물인화학식 2는 합성방법에 의해 합성하여 사용하였으며, 통상적인 모든 방법에 의해 얻을 수 있고, 시판되는 시약을 사용할 수 있다.

[0072] 또 하나의 양태로서, 본 발명은 상기 화학식 1 또는 화학식 1의 화합물 또는 이의 약학적으로 허용가능한 염을 포함하는, 염증성 질환 또는 암의 예방 및 치료용 약학 조성물에 관한 것이다.

[0073] 본 발명에 따른 화합물은 IL-6 또는 IL-11 에 의해 유도되는 신호전달체계를 억제하는 효과가 우수하므로, IL-6 또는 IL-11 에 의해 매개되는 질환, 특히 염증성 질환 또는 암 질환의 치료 또는 예방에 우수한 효과가 있다.

[0074] 상기 염증성 질환은 류마티스 관절염, 골다공증, 형질구 증가증, 초면역 글로불린 혈증, 빈혈, 신염, 악액질, 목축업자 질병, 맥관증식신염, 다발성 경화증, 포도막염, 만성 갑상선염, 지연과민증, 접촉피부염 아토피성 피부염, 전신성 홍반증, 크론병, 췌장염, 건선, 연소성 특발성 위축증, 당뇨병 및 알쯔하이머를 포함하나, 이에 제한되지 않으며, IL-6 또는 IL-11 에 매개되는 모든 염증성 질환을 포함한다. 또한, 상기 암 질환은 췌장암, 유방암, 전립선암, 뇌종양, 두경부암종, 흑색종, 골수종, 흑색종, 백혈병, 림프종, 간암, 위암, 결장암, 골암, 자궁암, 난소암, 직장암, 식도암, 소장암, 항문부근암, 결장암, 나팔관암종, 자궁내막암종, 자궁경부암종, 질암종, 음문암종, 호지킨병, 방광암, 신장암, 수뇨관암, 신장세포암종, 신장골반암종 및 중추신경계 종양을 포함하나, 이에 제한되지 않으며, IL-6 또는 IL-11 에 매개되는 모든 암 질환을 포함한다.

[0075] 본 발명의 약학조성물은, 조성물 총 중량에 대하여 상기 화합물을 0.0001 내지 10 중량%로, 바람직하게는 0.001 내지 1 중량%를 포함할 수 있다.

[0076] 또한, 본 발명의 옥사졸리딘계 화합물인 화학식 1과 인돌계 화합물인 화학식 2를 포함하는 조성물은 약학적 조성물의 제조에 통상적으로 사용하는 적절한 담체, 부형제 및 희석제를 더 포함할 수 있다.

[0077] 또한, 본 발명의 옥사졸리딘계 화합물인 화학식 1과 인돌계 화합물인 화학식 2의 약학적 투여 형태는 이들의 약학적 허용 가능한 염의 형태로도 사용될 수 있고, 또한 단독으로 또는 타 약학적 활성 화합물과 결합뿐만 아니라 적당한 집합으로 사용될 수 있다.

[0078] 본 발명에 따른 옥사졸리딘계 화합물인 화학식 1과 인돌계 화합물인 화학식 2를 포함하는 조성물은, 각각 통상의 방법에 따라 산제, 과립제, 정제, 캡슐제, 현탁액, 에멀젼, 시럽, 에어로졸 등의 경구형 제형, 외용제, 좌제 및 멸균 주사용액의 형태로 제형화하여 사용될 수 있다. 추출물을 포함하는 조성물에 포함될 수 있는 담체, 부형제 및 희석제로는 락토즈, 덱스트로즈, 수크로스, 솔비톨, 만니톨, 자일리톨, 에리스리톨, 말티톨, 전분, 아카시아 고무, 알지네이트, 젤라틴, 칼슘 포스페이트, 칼슘 실리케이트, 셀룰로즈, 메틸 셀룰로즈, 미정질 셀룰로스, 폴리비닐 피롤리돈, 물, 메틸히드록시벤조에이트, 프로필히드록시벤조에이트, 탈크, 마그네슘 스테아레이트 및 광물유를 들 수 있다. 제제화할 경우에는 보통 사용하는 충진제, 증량제, 결합제, 습윤제, 붕해제, 계면활성제 등의 희석제 또는 부형제를 사용하여 조제된다. 경구투여를 위한 고형제제에는 정제, 환제, 산제, 과립제, 캡슐제 등이 포함되며, 이러한 고형제제는 상기 추출물 또는 분획물에 적어도 하나 이상의 부형제 예를 들면, 전분, 칼슘카보네이트 (calcium carbonate), 수크로스(sucrose) 또는 락토오스(lactose), 젤라틴 등을 섞어 조제된다. 또한 단순한 부형제 이외에 마그네슘 스티레이트, 탈크 같은 윤활제들도 사용된다. 경구를 위한 액상 제제로는 현탁제, 내용액제, 유제, 시럽제 등이 해당되는 데 흔히 사용되는 단순희석제인 물, 리퀴드 파라핀 이외에 여러 가지 부형제, 예를 들면 습윤제, 감미제, 방향제, 보존제 등이 포함될 수 있다. 비경구 투여를 위한 제제에는 멸균된 수용액, 비수성용제, 현탁제, 유제, 동결건조 제제, 좌제가 포함된다. 비수성용제, 현탁제로는 프로필렌글리콜 (propylene glycol), 폴리에틸렌 글리콜, 올리브 오일과 같은 식물성 기름, 에틸올레이트와 같은 주사 가능한 에스테르 등이 사용될 수 있다. 좌제의 기제로는 위텝솔(witepsol), 마크로골, 트윈(tween) 61, 카카오지, 라우린지, 글리세로제라틴 등이 사용될 수 있다.

[0079] 본 발명의 옥사졸리딘계 화합물인 화학식 1과 인돌계 화합물인 화학식 2의 바람직한 투여량은 환자의 상태 및 체중, 질병의 정도, 약물형태, 투여경로 및 기간에 따라 다르지만, 당업자에 의해 적절하게 선택될 수 있다. 그러나, 바람직한 효과를 위해서, 본 발명의 추출물 또는 화합물은 1일 0.0001 내지 100 mg/kg으로, 바람직하게는 0.001 내지 100 mg/kg으로 투여하는 것이 좋다. 투여는 하루에 한번 투여할 수도 있고, 수회 나누어 투여할 수도 있다.

[0080] 또한, 본 발명의 약학 조성물은 쥐, 생쥐, 가축, 인간 등의 포유동물에 다양한 경로로 투여될 수 있다. 투여의

모든 방식은 예상될 수 있는데, 예를 들면, 경구, 직장 또는 정맥, 근육, 피하, 자궁내 경막 또는 뇌혈관내(intracerebroventricular) 주사에 의해 투여될 수 있다.

발명의 효과

[0081] 이상에서 설명한 바와 같이, 본 발명은 IL-6 및 IL-11에 의해 유도되는 신호전달체계의 저해 활성을 가지는 화학식 1 의 옥사졸리딘계 또는 화학식 2의 인돌계 화합물 또는 이의 약학적으로 허용되는 염, 또는 이들을 포함하는 조성물에 관한 것으로, 류마티스 관절염, 골다공증, 헝질구 증가증, 초면역 글로불린 혈증, 빈혈, 신염, 악액질, 목축업자 질병, 맥관증식신염, 다발성 경화증, 포도막염, 만성 갑상선염, 지연과민증, 접촉피부염 아토피성 피부염, 전신성 홍반증, 크론병, 췌장염, 건선, 연소성 특발성 위축증, 당뇨병 및 알쯔하이머을 포함하는 염증성 질환, 그리고 췌장암, 유방암, 전립선암, 뇌종양, 두경부암종, 흑색종, 골수종, 흑색종, 백혈병, 림프종, 간암, 위암, 결장암, 골암, 자궁암, 난소암, 직장암, 식도암, 소장암, 항문부근암, 결장암, 나팔관암종, 자궁내막암종, 자궁경부암종, 질암종, 음문암종, 호지킨병, 방광암, 신장암, 수뇨관암, 신장세포암종, 신장골반암종 및 중추신경계 종양을 포함하는 암의 예방 및 치료용 의약품으로 유용하게 사용될 수 있다.

도면의 간단한 설명

[0082] 도 1은 아이소프로필옥사졸리딘계 화합물과 인돌계 화합물이 HepG2 세포에서 IL-6로 유도되어지는 루시퍼라제(luciferase)의 발현 저해활성의 IC_{50}을 나타낸 도이다.

도 2는 본 발명에 따른 화학식 4d로 표시되는 아이소프로필옥사졸리딘계 화합물이 HepG2 세포에서 IL-11으로 유도되어지는 luciferase의 발현 저해활성을 나타낸 도이다.

도 3은 본 발명에 따른 화학식 4d로 표시되는 아이소프로필옥사졸리딘계 화합물이 HepG2 세포에서 IL-6 유도 STAT3 인산화 저해 활성을 나타낸 도이다.

도 4는 본 발명에 따른 화학식 4d로 표시되는 아이소프로필옥사졸리딘계 화합물이 U266 세포에서 IL-6 유도 JAK2 와 gp130 인산화 저해 활성을 나타낸 도이다.

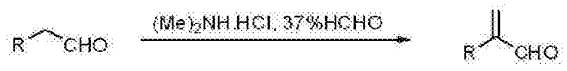
도 5는 본 발명에 따른 화학식 4d로 표시되는 아이소프로필옥사졸리딘계 화합물이 3T3L1 세포에서 인슐린 유도 아디포제네시스(adipogenesis)를 저해하는 IL-6 효과를 회복하는 활성을 나타낸 도이다.

발명을 실시하기 위한 구체적인 내용

[0083] 이하, 본 발명의 이해를 돕기 위하여 바람직한 실시예를 제시한다. 그러나 하기의 실시예는 본 발명을 보다 쉽게 이해하기 위하여 제공되는 것일 뿐, 실시예에 의해 본 발명의 내용이 한정되는 것은 아니다.

[0084] **실시예 : 화학식 1과 2로 표시되는 화합물의 합성**

[0085] **<실시예 1>**



1 2

1b ($R=CH(CH_3)_2$)	**2b** ($R=CH(CH_3)_2$)
1c ($R=C_4H_9$)	**2c** ($R=C_4H_9$)
1d ($R=C_5H_{11}$)	**2d** ($R=C_5H_{11}$)
1e ($R=C_6H_{13}$)	**2e** ($R=C_6H_{13}$)
1f ($R=C_7H_{15}$)	**2f** ($R=C_7H_{15}$)
1g (R=Bn)	**2g** (R=Bn)

[0086]

[0087] **3-메틸-2-메틸렌부타날의 제조(2b)**

[0088] 1b 아이소발리알디하이드 (500 mg, 58mmol, 1eq)에 포말알디하이드 수용액 (0.434ml, 58 mmol, 1eq, 37%)과 다이메틸아민하이드로클로라이드 용액을 (440mg, 50mmol, 0.93 eq) 첨가하여 70℃로 48시간 교반 하였다. 반응이 완결된 것을 TLC로 확인하였다. 모든 화합물이 2b가 된 것을 확인한 후에 물을 첨가하여 반응을 정지 시킨 후, 다이에틸이터를 첨가하여 유기층을 분리하였다. 유기층을 무수 황산마그네슘으로 건조시키고, 농축시킨 후, 노란색 액체물질 2b (44-62%)을 얻었다. 컬럼을 하지 않고 바로 다음 반응에 교반시켰다.

[0089] ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 9.52 (1H, s, -CHO), 6.23 (1H, s, -C=CHαHβ), 5.94 (1H, s, -C=CHαHβ), 2.78 (1H, m, $-CH(CH_3)_2$), 1.07 (6H, d, J = 6.8Hz, $-CH(CH_3)_2$)

[0090] **2-메틸렌헥사날의 제조(2c)**

[0091] 상기의 동일한 방법을 사용하여 노란색 액체물질의 2c (44-62%)를 얻었다.

[0092] ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 9.53 (1H, s, -CHO), 6.24 (1H, s, -C=CHαHβ), 5.98 (1H, s, -C=CHαHβ), 2.23 (2H, t, *J* = 7.3 Hz, $-CH_2CH_2CH_2CH_3$), 1.35-1.30 (4H, m, $-CH_2CH_2CH_2CH_3$), 0.90 (3H, t, *J* = 7.3Hz, $-CH_2CH_2CH_2CH_3$)

[0093] **2-메틸렌헥타날의 제조(2d)**

[0094] 상기의 동일한 방법을 사용하여 노란색 액체물질의 2d(44-62%)를 얻었다.

[0095] ^{1}H-NMR (CDC13, 500 MHz) δ 9.52 (1H, s, -CHO), 6.23 (1H, s, -C=CHαHβ), 5.97(1H, s, -C=CHαHβ), 2.21 (2H, t, *J* = 7.8Hz, $-CH_2CH_2CH_2CH_2CH_3$), 1.31-1.25 (6H, m, $-CH_2CH_2CH_2CH_2CH_3$), 0.87 (3H, t, *J* = 6.8Hz, $-CH_2CH_2CH_2CH_2CH_3$)

[0096] **2-메틸렌옥타날의 제조(2e)**

[0097] 상기의 동일한 방법을 사용하여 노란색 액체물질의 2e(65%)를 얻었다.

[0098] ^{1}H-NMR (CDC13, 500 MHz) δ 9.52 (1H, s, -CHO), 6.23 (1H, s, -C=CHαHβ), 5.97 (1H, s, -C=CHαHβ), 2.21 (2H, t, *J* = 7.8Hz, $-CH_2CH_2CH_2CH_2CH_2CH_3$), 1.31-1.25 (8H, m, $-CH_2CH_2CH_2CH_2CH_2CH_3$), 0.87 (3H, t, *J* = 6.8Hz, $-CH_2CH_2CH_2CH_2CH2CH_3$)

[0099] **2-메틸렌노네놀의 제조(2f)**

[0100] 상기의 동일한 방법을 사용하여 노란색 액체물질의 2f(44-62%)를 얻었다.

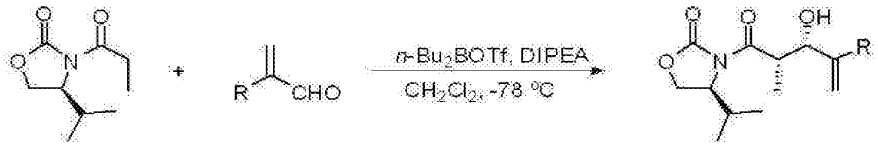
[0101] ^{1}H-NMR (CDC13, 500 MHz) δ 9.53 (1H, s, -CHO), 6.24 (1H, s, -C=CHαHβ), 5.98 (1H, s, -C=CHαHβ), 2.22 (2H, t, *J* = 7.3 Hz, $-CH_2(CH_2)_4CH_2CH_3$), 1.43 (2H, m, $-CH_2(CH_2)_4CH_2CH_3$), 1.18-1.30 (8H, m, $-CH_2(CH_2)_4CH_2CH_3$), 0.87 (3H, t, *J* = 6.8Hz, $-CH_2(CH_2)_4CH_2CH_3$).

[0102] **2-벤질아크릴알디하이드(2g)**

[0103] 상기의 동일한 방법을 사용하여 노란색 액체물질의 2g (51%)를 얻었다.

[0104] ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 9.56 (1H, s, -CHO), 7.25-7.10 (5H, m, Ar-H), 6.05 (1H, s, -C=CHαHβ), 6.01 (1H, s, -C=CHαHβ), 3.50 (2H, s, Ar-CH_2C=CH_2).

[0105] **<실시예 2>**



3a (S-이성질체) **2a** (R=CH_3) **4a** (R=CH3)
2b (R=$CH(CH_3)_2$) **4b** (R=$CH(CH_3)_2$)
2c (R=C_4H_9) **4c** (R=C_4H_9)
2d (R=C_5H_{11}) **4d** (R=C_5H_{11})
2e (R=C_6H_{13}) **4e** (R=C_6H_{13})
2f (R=C_7H_{15}) **4f** (R=C_7H_{15})
2g (R=Bn) **4g** (R=Bn)

[0106]

[0107] **(S)-3-((2S,3S)-3-하이드록시-2,4-디메틸펜트-4-에노일)-4-이소프로필옥사졸리딘-2-온의 제조(4a)**

[0108] 다이클로로메탄 (1.1 ml)에 용해시킨, (R)-(+)4-아이소프로필-3-프로피놀-2-옥사졸리디논 **3a** (100 mg, 0.541 mmol, 1eq)을 얼음수조에서 빙냉 시킨 후, 1M 다이클로로메탄에 용해되어져 있는 다이부틸보론 트리플로로메탄 썰퍼네이트(596, 0.596 mmo)와 다이아이소프로필에틸아민(90.6, 0.650mmol, 1.2eq)을 천천히 적가하여 같은 온도에서 50분 동안 교반하였다. 교반시킨 반응 혼합물을 영하 78℃에서 10분 동안 냉각시킨 후, 다이클로로메탄 (0.70 ml)에 용해시킨 메틸아크로렌 **2a** (59.0, 0.704 mmol)를 영하 78℃에서 30분 동안 교반하였다. 그 다음, 다시 얼음수조에서 빙냉 시킨 후 1시간 동안 교반한 후, pH 7.0 포스페이트 (1.2 ml), 메탄올 (1.1 ml), 30%wt 하이드로겐페록싸이드수용액(0.6 ml), 메탄올(1.1 ml)을 차례 대로 첨가하여 반응을 정지시킨다. 다시 얼음수조에서 1시간 더 교반시킨 후 다이클로로메탄과 물을 넣어 분리하여, 유기층을 분리하였다. 분리한 유기층을 소듐 클로라이드 수용액으로 씻어준 후, 무수 황산마그네슘으로 건조시키고, 농축시켰다. 얻어진 잔사를 실리카겔 컬럼크로마토그래피 (18 % n-헥산:에틸아세테이트)하여 물질을 분리하고 건조시켜 노란색 액체물질 **4a** (68mg, 49%)을 얻었다.

[0109] ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 5.12 (1H, s, -C=CHαHβ), 4.97 (1H, s, -C=CHαHβ), 4.48 (1H, m, -OCHαHβCHN), 4.40 (1H, brs, -CH(OH)), 4.30 (1H, dd, J = 17.1, 8.3 Hz, -OCHαHβCHN), 4.22 (1H, dd, J = 9.3, 2.9 Hz, -OCHαHβCHN), 3.97 (1H, dq, J = 7.3, 2.9 Hz, -CH(CH_3)CH(OH)-), 3.11 (1H, brs, J = 2.4 Hz, -OH), 2.39-2.32 (1H, m, -CH(CH_3)$_2$), 1.72 (s, 3H, -C(=CH)CH_3), 1.18 (3H, d, J = 6.8Hz, -CH(CH_3)CH(OH)-), 0.92 (3H, d, J = 6.8Hz, -CH(CH_3)$_2$), 0.89 (3H, d, J = 6.8Hz, -CH(CH_3)$_2$).

[0110] **(S)-3-((2S,3S)-3-하이드록시-2,5-디메틸-4-메틸렌헥사노일)-4-이소프로필옥사졸리딘-2-온의 제조(4b)**

[0111] 상기의 동일한 방법을 사용하여 노란색 액체물질의 **4b** (43%)를 얻었다.

[0112] ^{1}H-NMR (CDC13, 500 MHz) δ 5.19 (1H, s, -C=CHαHβ), 5.04 (1H, s, -C=CHαHβ), 4.48 (1H, m, -OCHαH$_\beta$CHN), 4.46 (1H, brs, -CH(OH)), 4.30 (1H, dd, J = 17.1, 8.3 Hz, -OCHαH$_\beta$CHN), 4.22 (1H, dd, J = 9.2, 2.9 Hz, -OCHαH$_\beta$CHN), 3.96 (1H, dq, J = 6.8, 2.9 Hz, -CH(CH_3)CH(OH)-), 3.08 (1H, brs, -OH), 2.38-2.32

(1H, m, -CH(CH_3)$_2$), 2.18-2.13 (1H, m, -C(=CH)CH(CH_3)$_2$), 1.19 (3H, d, J = 6.8Hz, -CH(CH_3)CH(OH)-), 1.09 (3H, d, J = 6.3 Hz, -C(=CH)CH(CH_3)$_2$), 1.05 (3H, d, J = 6.8Hz, -C(=CH)CH(CH_3)$_2$), 0.93 (3H, d, J = 6.8Hz, -CH(CH_3)$_2$), 0.89 (3H, d, J = 7.3Hz, -CH(CH_3)$_2$).

[0113] **(S)-3-((2S,3S)-3-하이드록시-2-메틸-4-메틸렌옥타노일)-4-이소프로필옥사졸리딘-2-온의 제조(4c)**

[0114] 상기의 동일한 방법을 사용하여 노란색 액체물질의 **4c** (16%)를 얻었다.

[0115] ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 5.16 (1H, s, -C=CHαHβ), 4.97 (1H, s, -C=CHαH$_\beta$), 4.46 (1H, m, -OCHαH$_\beta$CHN), 4.41 (1H, brs, -CH(OH)), 4.28 (1H, dd, J = 17.6, 9.2 Hz, -OCHαH$_\beta$CHN), 4.22 (1H, dd, J = 9.3, 2.9 Hz, -OCHαH$_\beta$CHN), 3.95 (1H, dq, J = 6.8, 2.9 Hz, -CH(CH_3)CH(OH)-), 3.13 (1H, brs, -OH), 2.38-2.33 (1H, m, -CH(CH_3)$_2$), 1.98 (2H, m, -C(=CH)CH_2CH$_2$CH$_2$CH$_3$), 1.46-1.40 (2H, m, -C(=CH)CH$_2$CH_2CH$_2$CH$_3$), 1.35-1.23 (2H, m, -C(=CH)CH$_2$CH$_2$CH_2CH$_3$), 1.17 (3H, d, J = 6.8Hz, -CH(CH_3)CH(OH)-), 0.91 (3H, d, J = 6.8Hz, -CH(CH_3)$_2$), 0.87 (3H, d, J = 7.3Hz, -CH(CH_3)$_2$), 0.86 (3H, d, J = 7.3Hz, -CH(CH_3)$_2$), 0.85 (3H, t, J = 5.6Hz, -C(=CH)CH$_2$CH$_2$CH$_2$CH_3).

[0116] **(S)-3-((2S,3S)-3-하이드록시-2-메틸-4-메틸렌노나노일)-4-이소프로필옥사졸리딘-2-온의 제조(4d)**

[0117] 상기의 동일한 방법을 사용하여 노란색 액체물질의 **4d** (84%)를 얻었다.

[0118] ^{1}H-NMR ($CDCl_3$, 500 MHz δ 5.18 (1H, s, -C=CHαHβ), 4.99 (1H, s, -C=CHαHβ), 4.50-4.47 (1H, m, -OCHαHβCHN), 4.42 (1H, brs, -CH(OH)), 4.28 (1H, dd, J = 17.4, 9.0 Hz, -OCHαHβCHN), 4.22 (1H, dd, J = 9.3, 2.9 Hz, -OCHαH$_\beta$CHN), 3.95 (1H, dq, J = 7.3, 2.9 Hz, -CH(CH_3)CH(OH)-), 3.15 (1H, brs, -OH), 2.39-2.33 (1H, m, -CH(CH_3)$_2$), 2.04-1.90 (2H, m, -C(=CH)CH_2CH$_2$CH$_2$CH$_2$CH$_3$), 1.49-1.43 (2H, m, -C(=CH)CH$_2$CH_2CH$_2$CH$_2$CH$_3$), 1.34-1.28 (4H, m, -C(=CH)CH$_2$CH$_2$CH$_2$CH_2CH$_3$), 1.18 (3H, d, J = 7.3Hz, -CH(CH_3)CH(OH)-), 0.93 (3H, d, J = 7.1 Hz, -CH(CH_3)$_2$), 0.88 (3H, d, J = 7.1 Hz, -CH(CH_3)$_2$), 0.88 (3H, t, J = 5.4 Hz, -C(=CH)CH$_2$CH$_2$CH$_2$CH$_2$CH_3).

[0119] **(S)-3-((2S,3S)-3-하이드록시-2-메틸-4-메틸렌데카노일)-4-이소프로필옥사졸리딘-2-온의 제조(4e)**

[0120] 상기의 동일한 방법을 사용하여 노란색 액체물질의 **4e** (87%)를 얻었다.

[0121] ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 5.18 (1H, s, -C=CHαHβ), 4.99 (1H, s, -C=CHαHβ), 4.50-4.47 (1H, m, -OCHαHβCHN), 4.42 (1H, brs, -CH(OH)), 4.28 (1H, dd, J = 17.4, 9.0 Hz, -OCHαHβCHN), 4.22 (1H, dd, J = 9.3, 2.9 Hz, -OCHαH$_\beta$CHN), 3.95 (1H, dq, J = 7.3, 2.9 Hz, -CH(CH_3)CH(OH)-), 3.15 (1H, brs, -OH), 2.39-2.33 (1H, m, -CH(CH_3)$_2$), 2.04-1.90 (2H, m, -C(=CH)CH_2CH$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 1.49-1.43 (2H, m, -C(=CH)CH$_2$CH_2CH$_2$CH$_2$CH$_2$CH$_3$), 1.34-1.28 (6H, m, -C(=CH)CH$_2$CH$_2$CH_2CH$_2$CH$_2$CH$_3$), 1.18 (3H, d, J = 7.3Hz, -CH(CH_3)CH(OH)-), 0.93 (3H, d, J = 7.1 Hz, -CH(CH_3)$_2$), 0.88 (3H, d, J = 7.1 Hz, -CH(CH_3)$_2$), 0.88 (3H, t, J = 5.4 Hz, -C(=CH)CH$_2$CH$_2$CH$_2$CH$_2$CH$_2$CH_3).

[0122] **(S)-3-((2S,3S)-3-하이드록시-2-메틸-4-메틸렌운데카노일)-4-이소프로필옥사졸리딘-2-온의 제조(4f)**

[0123] 상기의 동일한 방법을 사용하여 노란색 액체물질의 **4f** (41%)를 얻었다.

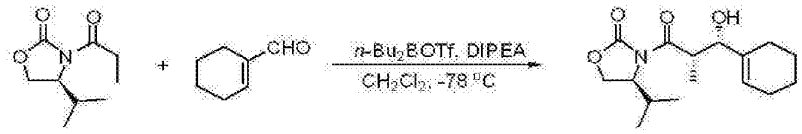
[0124] ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 5.18 (1H, s, -C=CHαHβ), 4.99 (1H, s, -C=CHαHβ), 4.50-4.46 (1H, m, -OCHαHβCHN), 4.42 (1H, brs, -CH(OH)), 4.30 (1H, dd, *J* = 17.4, 9.1 Hz, -OCHαHβCHN), 4.23 (1H, dd, *J* = 9.1, 3.1 Hz, -OCHαHβCHN), 3.95 (1H, dq, *J* = 7.1, 2.8 Hz, -CH(CH_3)CH(OH)-), 3.12 (1H, brs, -OH), 2.37-2.33 (1H, m, -CH(CH3)2), 1.95 (2H, m, -C(=CH)CH_2(CH_2)$_5CH_3$), 1.19-1.26 (10H, m, -C(=CH)CH_2(CH_2)$_5CH_3$), 1.18 (3H, d, *J* = 7.1 Hz, -CH(CH_3)CH(OH)-), 0.93 (3H, d, *J* = 7.0 Hz, -CH(CH_3)$_2$), 0.89 (3H, d, *J* = 6.72 Hz, -CH(CH_3)$_2$), 0.86 (3H, t, *J* = 5.8 Hz, -C(=CH)CH_2(CH_2)$_5CH_3$).

[0125] **(S)-3-((2S,3S)-4-벤질-3-하이드록시-2-메틸펜트-4-에노일)-4-이소프로필옥사졸리딘-2-온의 제조(4g)**

[0126] 상기의 동일한 방법을 사용하여 노란색 액체물질의 **4g**(72%)를 얻었다.

[0127] ^{1}H-NMR ($CDCl_3$, 500 MHz) : δ 7.28 (5H, m, Ar-H), 5.33 (1H, s, -C=CHαHβ), 4.98 (1H, s, -C=CHαHβ), 4.42-4.41 (1H, m, -OCHαHβCHN), 4.39 (1H, d, *J*=3.3Hz, -CH(OH)), 4.25 (1H, dd, *J*=9.0,8.3Hz, -OCHαHβCHN), 4.19 (1H,dd,*J*=9.0,3.2Hz,-OCHαHβCHN), 4.01 (3H, dq, *J*=7.1,3.0Hz, -CH(CH_3)CH(OH)-), 3.44 (1H, d, *J*=15.4Hz, -C(=CH)CHαHβPh), 3.30 (1H, d, *J*=15.4Hz, -C(=CH)CHαHβPh), 3.14 (1H, brs, -OH) ,2.35-2.29 (1H, m, -CH(CH_3)$_2$), 1.22 (3H, d, *J*=7.1Hz, -CH(CH_3)CH(OH)-), 0.91(3H, d, *J*=7.1Hz,-CH(CH_3)$_2$), 0.87 (3H, d, *J*=6.8Hz, -CH(CH_3)$_2$).

[0128] **<실시예 3>**



3a **2h** **4h**

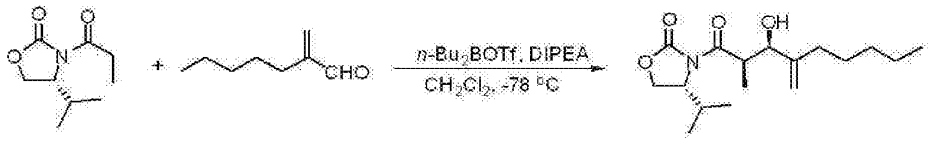
[0129]

[0130] 다이클로로메탄 (1.5 ml)에 용해시킨, (R)-(+)4-아이소프로필-3-프로피놀-2-옥사졸리디논 **3a** (100 mg, 0.541 mmol, 1 eq)을 얼음수조에서 빙냉 시킨 후, 1M 다이클로로메탄에 용해되어져 있는 다이부틸보론 트리플로로메탄 썰퍼네이트 (596 , 0.596 mmo)와 다이아이소프로필에틸아민 (90.6 , 0.650 mmol, 1.2 eq) 을 천천히 적가하여 같은 온도에서 50분 동안 교반하였다. 교반시킨 반응 혼합물을 영하 78℃ 에서 10분 동안 냉각시킨 후, 다이클로로메탄 (0.70 ml)에 용해시킨 1-싸이클로헥센 카복실알디하이드 **2h** (92.25, 0.81 mmol)를 영하 78℃에서 30분 동안 교반하였다. 그 다음, 다시 얼음수조에서 빙냉 시킨 후 1시간 동안 교반한 후, pH 7.0 포스페이트 (1.2 ml), 메탄올 (1.1 ml), 30%wt 하이드로겐페록싸이드수용액 (0.6 ml), 메탄올(1.1 ml)을 차례대로 첨가하여 반응을 정지시킨다. 다시 얼음수조에서 1시간 더 교반시킨 후 다이클로로메탄과 물을 넣어 분리하여, 유기층을 분리하였다. 분리한 유기층을 소듐클로라이드 수용액으로 씻어준 후, 무수 황산마그네슘으로 건조시키고, 농축시켰다. 얻어진 잔사를 실리카겔 컬럼크로마토그래피 (18 % n-헥산:에틸아세테이트)하여 물질을 분리하고 건조시켜 노란색 액체물질 **4h** (22.3 mg 14.8 %)를 얻었다.

[0131] ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 5.81 (1H, t, *J* = 3. 4 Hz ,Cyclo = (CH_2)), 4.50-4.47 (1H, m, -OCHαHβCHN), 4.42 (1H, brs, -CH(OH)), 4.28 (1H, dd, *J* = 17.4, 9.0 Hz, -OCHαHβCHN), 4.22 (1H, dd, *J* = 9.3, 2.9 Hz, -OCHαHβCHN), 3.95 (1H, dq, *J* = 7.3, 2.9 Hz, -CH(CH_3)CH(OH)-), 2.93 (1H, brs, -OH), 2.40-2.33 (1H, m, -CH(CH_3)$_2$), 2.07-2.05 (2H, m, -Cyclo(CH_2)), 1.927 (2H, t, *J* = 17.60 Hz, -Cyclo(CH_2)), 1.67-1.55 (4H, m, -Cyclo(CH_2CH_2), 1.18 (3H, d, *J* = 7.3 Hz, -CH(CH_3)CH(OH)-), 0.93 (3H, d, *J* = 7.1 Hz, -CH(CH_3)$_2$), 0.88

(3H, d, J = 7.1 Hz, -CH(CH_3)$_2$).

[0132] <실시예 4>



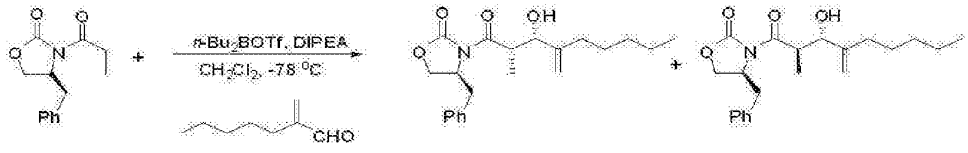
3b (R-이성질체) 2d 5

[0133]

[0134] 다이클로로메탄 (2.0 ml)에 용해시킨, (R)-(-)4-아이소프로필-3-프로피놀-2-옥사졸리디논 **3b** (100 mg, 0.541 mmol, 1 eq)을 얼음수조에서 빙냉 시킨 후, 1M 다이클로로메탄에 용해되어져 있는 다이부틸보론 트리플로로메탄 썰퍼네이트 (596 , 0.596 mmo)와 다이아이소프로필에틸아민 (90.6 , 0.650 mmol, 1.2 eq) 을 천천히 적가하여 같은 온도에서 50분 동안 교반하였다. 교반 시킨 반응 혼합물을 영하 78℃에서 10분 동안 냉각시킨 후, 다이클로로메탄 (0.70 ml)에 용해시킨 2-메틸렌헵탈 **2d** (68.14 , 1.07 mmol)를 영하 78℃에서 30분 동안 교반하였다. 그 다음, 다시 얼음수조에서 빙냉 시킨 후 1시간 동안 교반한 후, pH 7.0 포스페이트 (0.1 ml), 메탄올 (0.2 ml), 30%wt 하이드로겐페록싸이드수용액(0.2 ml), 메탄올(0.2 ml)을 차례대로 첨가하여 반응을 정지시킨다. 다시 얼음수조에서 1시간 더 교반시킨 후 다이클로로메탄과 물을 넣어 분리하여, 유기층을 분리하였다. 분리한 유기층을 소듐클로라이드 수용액으로 씻어준 후, 무수 황산마그네슘으로 건조시키고, 농축시켰다. 얻어진 잔사를 실리카겔 컬럼크로마토그래피 (15 % n-헥산:에틸아세테이트)하여 물질을 분리하고 건조시켜 노란색 액체물질 **5** (146.3 mg 87.2 %)를 얻었다.

[0135] ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 5.18 (1H, s, -C=CHαHβ), 4.99 (1H, s, -C=CHαHβ), 4.50-4.47 (1H, m, -OCHαHβCHN), 4.42 (1H, brs, -CH(OH)), 4.28(1H, dd, J = 17.4,9.0 Hz, -OCHαHβCHN), 4.22 (1H, dd, J = 9.3, 2.9 Hz, -OCHαH$_\beta$CHN), 3.95 (1H, dq, J = 7.3, 2.9 Hz, -CH(CH_3)CH(OH)-), 3.15 (1H, brs, -OH), 2.39-2.33 (1H, m, -CH(CH_3)$_2$), 2.04-1.90 (2H, m, -C(=CH)$CH_2CH_2CH_2CH_2CH_3$), 1.49-1.43 (2H, m, -C(=CH)$CH_2CH_2CH_2CH_2CH_3$), 1.34-1.28 (4H, m, -C(=CH)$CH_2CH_2CH_2CH_2CH_3$), 1.18 (3H, d, J = 7.3Hz, -CH(CH_3)CH(OH)-), 0.93 (3H, d, J = 7.1Hz, -CH(CH_3)$_2$), 0.88 (3H, d, J=7.1Hz,-CH(CH_3)$_2$), 0.88 (3H, t, J = 5.4 Hz, -C(=CH)$CH_2CH_2CH_2CH_2CH_3$).

[0136] <실시예 5>



6 7a (9 : 1) 7b

[0137]

[0138] 다이클로로메탄 (1.5 ml)에 용해 시킨, (R)-4-벤질-3-프로피오닐옥사졸리딘-2-온 **6** (100 mg, 0.43 mmol, 1 eq)을 얼음수조에서 빙냉 시킨 후, 1M 다이클로로메탄에 용해되어져 있는 다이부틸보론 트리플로로메탄 썰퍼네이트 (515 , 0.51 mmol)와 다이아이소프로필에틸아민 (104 , 0.60 mmol, 1.4 eq) 을 천천히 적가 하여 같은 온도에서 50분 동안 교반하였다. 교반 시킨 반응 혼합물을 영하 78℃에서 10분 동안 냉각시킨 후, 다이클로로메탄 (0.70 ml)에 용해시킨 2-메틸렌헵탈 **2d** (68.14 , 1.07 mmol)를 영하 78℃에서 30분 동안 교반하였다. 그 다음, 다시 얼음수조에서 빙냉 시킨 후 1시간 동안 교반한 후, pH 7.0 포스페이트 (0.1 ml), 메탄올 (0.2 ml), 30%wt 하이드로겐페록싸이드수용액 (0.2 ml), 메탄올 (0.2 ml)을 차례대로 첨가하여 반응을 정지시킨다. 다시 얼음수조에서 1시간 더 교반시킨 후 다이클로로메탄과 물을 넣어 분리하여, 유기층을 분리하였다. 분리한 유기층을 소듐클로라이드 수용액으로 씻어준 후, 무수 황산마그네슘으로 건조시키고, 농축시켰다. 얻어진 잔사를 실리카겔

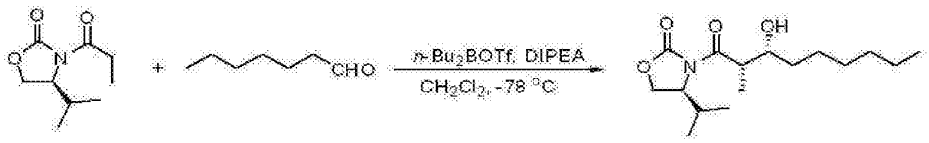
컬럼크로마토그래피 (18 % n-헥산:에틸아세테이트)하여 물질을 분리여 건조시켜 노란색 액체물질 **7a** (105mg, 68%)를 얻었다.

[0139] ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 7.36-7.33 (2H, m, Ar-**H**), 7.30-7.27 (1H, m, Ar-**H**), 7.22-7.21 (2H, m, Ar-**H**), 5.19 (1H, s, -C=C**H**αH$_\beta$), 5.00 (1H, s, -C=CHαH$_\beta$), 4.74-4.69 (1H, m, -OCHαH$_\beta$CHN), 4.45 (1H, brs, -C**H**(OH)), 4.23 (2H, m, PhCH_2-), 3.96 (1H, dd, *J* = 10.2, 3.4 Hz, -OCHαH$_\beta$C**H**N), 3.27 (1H, dd, *J* = 13.2, 3.4 Hz, -OCHαH$_\beta$CHN), 3.01 (1H, brs, -OH), 2.81 (1H, dd, *J* = 13.2, 9.2 Hz, -C**H**(CH$_3$)CH(OH)-), 2.00 (2H, m, -C(=CH)CH_2(CH$_2$)$_2$CH$_2$CH$_3$), 1.48 (2H, m, -C(=CH)CH$_2$(CH$_2$)$_2$CH_2CH$_3$), 1.32 (4H, m, -C(=CH)CH$_2$(CH_2)$_2$CH$_2$CH$_3$), 1.20 (3H, d, *J* = 6.8 Hz, -CH(CH_3)CH(OH)-), 0.90 (3H, t, *J* = 6.8Hz, -C(=CH)CH$_2$(CH$_2$)$_2$CH$_2$CH_3)

[0140] **실시예5**에서 얻은 잔사를 실리카겔컬럼크로마토그래피(18 % n-헥산:에틸아세테이트)에서 7a를 얻은 뒤, (12 % n-헥산:에틸아세테이트)에 **7b** (21mg, 14%)를 얻었다.

[0141] ^{1}H-NMR ($CDCl_3$, 500 MHz) : δ7.36-7.33 (2H, m, Ar-**H**), 7.30-7.27 (1H, m, Ar-**H**), 7.22-7.21 (2H, m, Ar-**H**), 5.19 (1H, s, -C=C**H**αH$_\beta$), 5.00 (1H, s, -C=CHαH$_\beta$), 4.74-4.69 (1H, m, -OCHαH$_\beta$CHN), 4.45 (1H, brs, -C**H**(OH)), 4.26-4.19 (2H, m, PhCH_2-), 3.95 (1H, dd, *J*=10.2, 3.4 Hz, -OCHαH$_\beta$C**H**N), 3.27 (1H, dd, *J* = 13.7, 3.6 Hz, -OCHαH$_\beta$CHN), 3.01 (1H, brs, -O**H**), 2.81 (1H, dd, *J*=13.2, 9.8 Hz, -C**H**(CH$_3$)CH(OH)-), 2.00 (2H, m, -C(=CH)CH_2(CH$_2$)$_2$CH$_2$CH$_3$), 1.48 (2H, m, -C(=CH)CH$_2$(CH$_2$)$_2$CH_2CH$_3$), 1.32 (4H, m, -C(=CH)CH$_2$(CH_2)$_2$CH$_2$CH$_3$), 1.20 (3H, d, *J* = 6.8 Hz, -CH(CH_3)CH(OH)-), 0.90 (3H, t, *J* = 6.8 Hz, -C(=CH)CH$_2$(CH$_2$)$_2$CH$_2$CH_3).

[0142] **<실시예 6>**



3a **8** **9**

8(R1 = C6H13) **9 (R1=C6H13)**

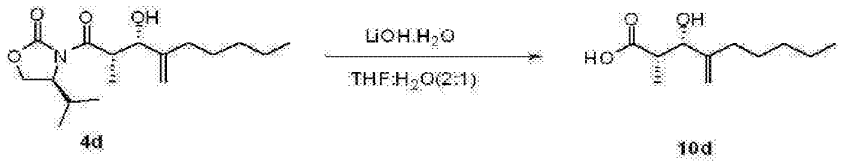
[0143]

[0144] 다이클로로메탄 (1.5 ml)에 용해시킨, (S)-(+)4-아이소프로필-3-프로피놀-2-옥사졸리디논 **3a** (100 mg, 0.541 mmol, 1 eq)을 얼음수조에서 빙냉시킨 후, 1M 다이클로로메탄에 용해되어져 있는 다이부틸보론 트리플로로메탄 썰퍼네이트 (596 , 0.596 mmol)와 다이아이소프로필에틸아민 (90.6 , 0.650 mmol, 1.2eq) 을 천천히 적가하여 같은 온도에서 50분 동안 교반하였다. 교반 시킨 반응 혼합물을 영하 78° C 에서 10분 동안 냉각시킨 후, 다이클로로메탄 (0.70 ml)에 용해시킨 헥타날 **8** (92.25 , 0.81 mmol)를 영하 78℃에서 30분 동안 교반하였다. 그 다음, 다시 얼음수조에서 빙냉 시킨 후 1시간 동안 교반 한 후, pH 7.0 포스페이트 (1.2 ml), 메탄올 (1.1 ml), 30%wt 하이드로겐 페록싸이드수용액 (0.6 ml), 메탄올 (1.1 ml)을 차례대로 첨가하여 반응을 정지시킨다. 다시 얼음수조에서 1시간 더 교반 시킨 후 다이클로로메탄과 물을 넣어 분리하여, 유기층을 분리하였다. 분리한 유기층을 소듐클로라이드 수용액으로 씻어준 후, 무수 황산마그네슘으로 건조시키고, 농축시켰다. 얻어진 잔사를 실리카겔 컬럼크로마토그래피 (18 % n-헥산:에틸아세테이트)하여 물질을 분리하고 건조시켜 노란색 액체물질 **9** (22.3 mg, 14.8%)를 얻었다.

[0145] ^{1}H-NMR ($CDCl_3$, 500 MHz) : δ 5.54 (1H, t, *J* = 7.3 Hz, -CH(CH$_3$)C**H**(O**H**), 4.48 - 4.41 (1H, m, -OCHαH$_\beta$

CHN), 4.39 (1H, brs, -CH(OH)), 4.28 (1H, dd, J = 17.6, 9.3 Hz, -OCHαHβCHN), 4.23 (1H, dd, J = 7.8, 2.9Hz, -OCHαHβCHN), 3.94 (1H, dq, J = 6.8, 2.9 Hz, -CH(CH_3)CH(OH)-), 2.38 - 2.33 (1H, m, -CH(CH_3)$_2$), 2.10 - 2.01 (3H, m, -CHαHβCH_2(CH_2)$_3CH_3$), 1.82 - 1.76 (1H, m, -CHαHβCH_2(CH_2)$_3CH_3$), 1.37 - 1.27 (6H, m, -CHαHβCH_2(CH_2)$_3CH_3$), 1.17 (3H, d, J = 7.3Hz, -CH(CH_3)CH(OH)-), 0.92 (3H, d, J = 6.8 Hz, -CH(CH_3)$_2$), 0.90 (3H, d, J = 7.3 Hz, -CH(CH_3)$_2$), 0.87 (3H, t, J = 5.8 Hz, -CHαHβCH_2(CH_2)$_3CH_3$).

[0146] <실시예 7>

[0147] 

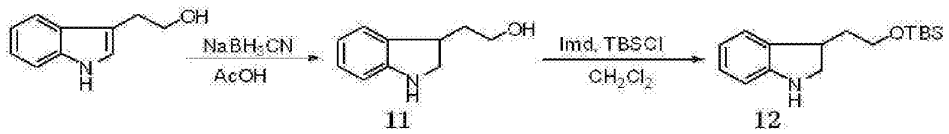
4d 10d

[0148] (2S,3S)-3-하이드록시-2-메틸-4-메틸렌노나노익 애시드의 제조(10d)

[0149] 실시예 2에서 제조된 (S)-3-((2S,3S)-3-하이드록시-2-메틸-4-메틸렌노나노일)-4-이소프로필옥사졸리딘-2-온 4d(948 mg, 3.05 mmol)를 테트라하이드로퓨란 : 물 (27 ml : 27 ml)의 혼합물에 용해시키고, 상온에서 리튬히드록사이드(256 mg, 6.10 mmol)를 첨가하였다. 상온에서 12시간 동안 교반하여 반응을 완료시켰다. 감압 하에서 테트라하이드로퓨란을 제거하고 pH 7까지 중화시킨 후 반응혼합물에 에틸아세테이트를 첨가하여 유기층을 분리하였다. 분리한 유기층을 소듐클로라이드 수용액으로 씻어준 후, 무수 황산마그네슘으로 건조시키고, 농축시켰다. 얻어진 액체물질 10d (647 mg, 106.1 %)를 바로 다음 반응에 첨가시켰다.

[0150] ^{1}H-NMR ($CDCl_3$, 500 MHz) : δ 5.10 (1H, s, -C=CHαHβ), 4.99 (1H, s, -C=CHαHβ), 4.52 (1H, d, J = 3.4 Hz, -CH(CH_3)CH-OH), 2.71 (1H, m, -CH(CH3)CH-OH), 2.02-1.93 (2H, m, -$CH_2CH_2CH_2CH_2CH_3$), 1.48 (2H, m, -$CH_2CH_2CH_2CH_2CH_3$), 1.31 (4H, m, -$CH_2CH_2CH_2CH_2CH_3$), 1.14 (3H, d, J = 37.3 Hz, -CH(CH_3)CH-OH), 0.90 (3H, t, J = 6.8 Hz, -$CH_2CH_2CH_2CH_2CH_3$).

[0151] <실시예 8>

[0152] 

11 12

[0153] 2-(인돌린-3-일)에탄올의 제조(11)

[0154] 아세틱에시드 (41.5 ml)에 용해 된 2-(3-인돌)-에탄올 (1.2 g, 7.06 mmol)을 얼음 수조에 넣어 빙냉시킨 후 소듐싸이아노보로하이드라이드 (2.2 g, 35.34 mmol)를 첨가하였다. 얼음 수조를 빼고 상온에서 3시간 동안 교반하였다. 반응이 완결된 것을 확인하고, 포화상태의 소듐바이오카보네이트 수용액을 첨가하여 반응을 정지 시켰다. 반응 혼합물을 중화시킨 다음, 에틸아세테를 넣어 유기층을 분리 하였다. 분리한 유기층을 소듐클로라이드 수용액으로 씻어준 후, 무수 황산마그네슘으로 건조시키고, 농축시켰다. 얻어진 잔사를 실리카겔 컬럼크로마토그래피 (n-헥산 : 에틸아세테이트 = 10 : 1)하여 물질을 분리하고 건조시켜 노란색 액체물질 11 (432 mg 37.4 %)을 얻었다.

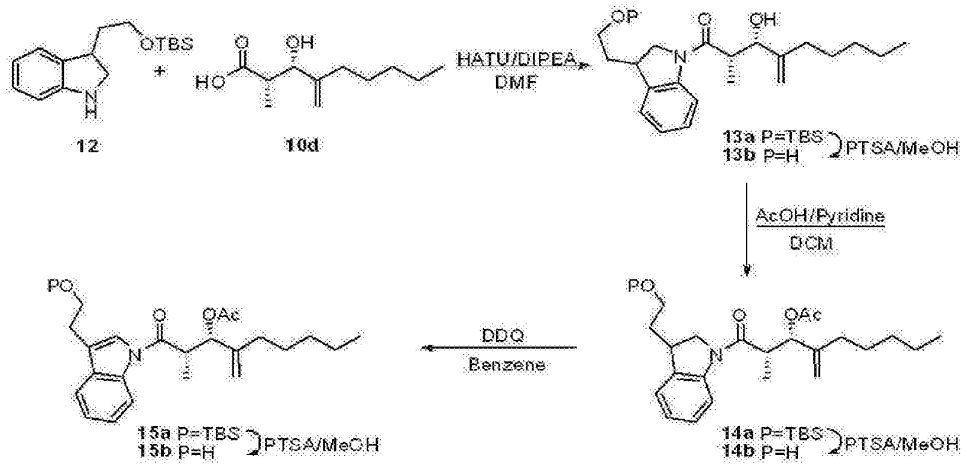
[0155] ^{1}H-NMR($CDCl_3$, 500 MHz) : δ 7.10 (1H, d, J = 7.9 Hz), 7.06 (1H, dd, J = 7.9, 7.3 Hz), 6.76 (1H, dd, J = 7.6, 7.3 Hz), 6.68 (1H, d, J = 7.6 Hz), 3.74 - 3.55 (3H, m), 3.44 (1H, m), 3.28 (1H, dd, J = 8.6, 5.9 Hz), 3.10 (1H, brs), 2.09 (1H, m), 1.79 (1H, m)

[0156] **3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린의 제조(12)**

[0157] 2-(인돌린-3-일)에탄올 **11** (365 mg, 2.23 mmol)을 다이클로로메탄에 녹인 후, 빙냉 시킨 얼음 수조에서 이미다졸 (182.6 mg, 2.68 mmol)과 터셔리-부틸다이메틸실릴 클로라이드 (404.2 mg, 2.68 mmol)를 차례대로 첨가하였다. 상온에서 3시간 동안 교반 시킨 후, 반응이 완결된 것을 확인하였다. 반응 혼합물에 물과 다이클로로메탄에 넣어 반응을 중지시킨 후, 유기층을 분리하였다. 분리한 유기층을 소듐클로라이드 수용액으로 씻어준 후, 무수 황산마그네슘으로 건조시키고, 농축시켰다. 얻어진 잔사를 실리카겔 컬럼크로마토그래피 (n-헥산 : 에틸아세테이트 = 20 : 1) 하여 물질을 분리하고 건조시켜 노란색 액체물질 **12** (570.5 mg, 92.8 %)를 얻었다.

[0158] ^{1}H-NMR ($CDCl_3$, 500 MHz) : δ 7.10 (1H, d , J = 7.9 Hz), 7.06 (1H, dd, J = 7.9, 7.3 Hz), 6.76 (1H, dd, J = 7.6, 7.3 Hz), 6.68 (1H, d, J = 7.6 Hz), 3.74 - 3.55 (3H, m), 3.44 (1H, m), 3.28 (1H, dd, J = 8.6, 5.9 Hz), 2.09 (1H, m), 1.79 (1H, m), 0.92 (9H, s, -Si-$(CH_3)_2$-$(CH_3)_3$), 0.09 (6H, s, -Si-$(CH_3)_2$-$(CH_3)_3$).

[0159] **<실시예 9>**



[0160]

[0161] **(2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-3-하이드록시-2-메틸-4-메틸렌노난-1-온의 제조(13a)**

[0162] 3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린 **12** (405 mg, 1.45 mmol) ,(2S, 3S)-3-하이드록시-2-메틸-4-메틸레노넥스 에스드 (438 mg, 2.18 mmol) 및 O-(7-아자벤조트리아졸-1-일)-N,N,N`,N`-테트라메틸-우라늄 (832.4 mg, 2.18 mmol) 을 디메틸포름아미드 20.0 ml에 용해시키고, N,N-디이소프로필에틸아민(0.17 ml, 1.0 mmol)을 첨가하였다. 상온에서 교반시키고, 에틸아세테이트 및 염화나트륨 수용액으로 분리시키고, 유기층을 무수 황산마그네슘으로 건조시키고, 농축시킨 후, 실리카겔 칼럼 크로마토그래피(n-헥산 : 에틸아세테이트 = 10 : 1)로 정제하여 목적 화합물**13a** (404 mg, 60.2 %)**를** 오일상태로 얻었다.

[0163] ^{1}H-NMR ($CDCl_3$, 500 MHz) : δ 8.22 (1H, d, J = 8.3 Hz, Ar-**H**), 7.22 (2H, m, Ar-**H**), 7.09 (1H, t, J = 7.3 Hz, Ar-**H**), 5.27 (1H, s, -C=C**H**αHβ), 5.02 (1H, s, -C=CHα**H**β), 4.50 (1H, brs, HC-O**H**), 4.27 (1H, t, J = 6.35 Hz, -**CH**αHβ-OTBS), 3.92 (1H, q, J = 5.86, 4.4 Hz, -CHα**H**β-OTBS), 3.87-3.76 (2H, m, -N-CH_2), 3.56 - 3.53 (1H, m, -CO-**CH**(CH_3), 2.80 (1H, m, Ar-**CH**), 2.01 (2H, m, -CH_2-CH_2-OTBS), 2.91 (1H, m, -C=CH-**CH**αHβ-CH_2), 1.82 (1H, m, -C=CH-CHα**H**β-CH_2), 1.51 (2H, m, -CH_2-CH_3), 1.34-1.32 (4H, m, -$CH_2CH_2CH_2CH_3$), 1.21 (3H, d, J = 6.84 Hz, C=O-CH(CH_3), 1.19 (3H, t, J = 7.33 Hz, CH_2CH_3), 0.90 (9H, s, -Si-$(CH_3)_2$-$(CH_3)_3$), 0.09 (6H, s, -Si-$(CH_3)_2$-$(CH_3)_3$).

[0164] **(2S,3S)-3-하이드록시-1-(3-(2-하이드록시에틸)인돌린-1-일)-2-메틸-4-메틸렌노난-1-온의 제조(13b)**

[0165] (2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-3-하이드록시-2-메틸-4-메틸렌노난-1-온 **13a** (54.5 mg, 0.12 mmol)을 테트로하이드로퓨란 (5 ml)에 용해 시킨 후, 테트로하이드로퓨란에 1.0 M로 녹여져 있는 테트라부틸암모니움 플로라이드 (0.15 ml)를 얼음 수조 상태에서 첨가하였다. 상온에서 2시간 동안 교반 한 후, 반응이 완결된 것을 확인하였다. 반응 혼합물에 메탄올을 넣어 반응을 중지 시킨 후, 얻어진 잔사를 바로 실리카겔 컬럼크로마토그래피 (n-헥산:에틸아세테이트 = 1 : 1)하여 물질을 분리하고 건조시켜 목적화합물 **13b** (39 mg, 95 %)를 오일상태로 얻었다.

[0166] ^{1}H-NMR (CDCl$_3$, 500 MHz) : δ 8.25 (1H, d, *J* = 8.3 Hz, Ar-**H**), 7.25 (2H, m, Ar-**H**), 7.08 (1H, t, *J* = 6.8 Hz, Ar-**H**), 5.27 (1H, s, -C=C**H**αHβ), 5.02 (1H, s, -C=CHα**H**β), 4.50 (1H, brs, HC-**OH**), 4.49 (2H, s, HC-**OH**), 4.28 (1H, q, *J* = 6.35 Hz, -CHαHβ-**OH**), 3.89 (1H, q, *J* = 5.86, 4.4 Hz, -CHαHβ-**OH**), 3.83 - 3.82 (2H, m, -N-**CH$_2$**), 3.79 (1H, brs, CH$_2$CH$_2$**OH**), 3.63 - 3.59 (1H, m, -CO-**CH**(CH$_3$), 2.78 (1H, m, Ar-**CH**), 2.06 (2H, m, -**CH$_2$**-CH$_2$-OH), 1.88 (1H, m, -C=CH-CH**α**H**β**-CH$_2$), 1.86 (1H, m, -C=CH-CHαHβ-**CH$_2$**), 1.50 (2H, m, -**CH$_2$**-CH$_3$), 1.34-1.32 (4H, m, -**CH$_2$CH$_2$CH$_2$**CH$_3$), 1.21 (3H, d, *J* = 7.3 Hz, C=O-CH(**CH$_3$**), 0.91 (3H, t, *J* = 6.84 Hz, CH$_2$**CH$_3$**).

[0167] **(2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-2-메틸-4-메틸렌-1-옥소노난-3-일 아세테이트의 제조(14a)**

[0168] 디클로로메탄으로 녹인 (2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-3-하이드록시-2-메틸-4-메틸렌노난-1-온 **13a** (357 mg, 0.77 mmol)에 피리딘 (0.34 ml, 3.88 mmol)을 첨가 한 후, 아세틱언하이드로스를 천천히 적가 하였다. 상온에서 12시간 동안 교반하였는데, 출발물질이 조금 남아 있어 4-다이메틸아미노피리딘 (1 eq) 첨가하여 상온에서 2시간 더 교반하였다. 반응이 완결된 것을 확인하고, 감압기로 피리딘을 제거한 뒤, 에틸아세테이트와 물을 첨가하여 유기층을 분리하였다. 분리한 유기층을 소듐클로라이드 수용액으로 씻어준 후, 무수 황산마그네슘으로 건조시키고, 농축시켰다. 얻어진 잔사를 실리카겔 컬럼크로마토그래피 (n - 헥산 : 에틸아세테이트 = 20 : 1)하여 물질을 분리하고 건조시켜 목적화합물 **14a** (570.5 mg, 92.8 %)를 오일상태로 얻었다.

[0169] ^{1}H-NMR (CDCl$_3$, 500 MHz) : δ 8.11 (1H, d, *J* = 7.8 Hz, Ar-**H**), 7.10 (2H, m, Ar-**H**), 6.95 (1H, t, *J* = 7.3 Hz, Ar-**H**), 5.42 (1H, d, *J* = 8.3 Hz, -CHOCOCH$_3$), 5.04 (1H, s, -C=C**H**αHβ), 4.84 (1H, s, -C=CHα**H**β), 4.14 (1H, t, *J* = 9.2 Hz, -CHαHβ-OTBS), 3.76 - 3.68 (2H, m, -N-**CH$_2$**), 3.69 (1H, t, *J* = 6.3Hz, -CHαHβ-OTBS), 3.44 (1H, m, -CO-**CH**(CH$_3$), 2.94 (1H, m, Ar-**CH**), 2.01 (3H, s, CHOCOCH$_3$), 1.96 (2H, m, -**CH$_2$**-CH$_2$-OTBS), 1.66 (1H, m, -C=CH-CH**α**H**β**-CH$_2$), 1.37 (1H, m, -C=CH-CHαHβ-**CH$_2$**), 1.17 (2H, m, -**CH$_2$**-CH$_3$), 0.86 (4H, m, -**CH$_2$CH$_2$CH$_2$**CH$_3$), 0.85 (9H, s, -Si-(CH$_3$)$_2$-(CH$_3$)$_3$), 0.85 (3H, d, *J* = 6.84 Hz, C=O-CH(**CH$_3$**), 0.76 (3H, t, *J* = 7.33 Hz, CH$_2$**CH$_3$**), 0.01 (6H, s, -Si-(**CH$_3$**)$_2$-(CH$_3$)$_3$).

[0170] **(2S,3S)-1-(3-(2-하이드록시에틸)인돌린-1-일)-2-메틸-4-메틸렌-1-옥소노난-3-일 아세테이트의 제조(14b)**

[0171] (2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-2-메틸-4-메틸렌-1-옥소노난-3-일 아세테이트 **14a** (64.4 mg, 0.12 mmol)을 메탄올 (3 ml)에 용해시킨 후, 피리딘 파-톨루엔-썰포네이트를 소량 넣어 준 후 상온에서 2시간 동안 교반하였다. 반응이 완결된 것을 확인한 후, 감압기로 메탄올을 날린 뒤 바로 실리카겔 컬럼크로마토그래피 (n - 헥산 : 에틸아세테이트 = 1 : 1)하여 물질을 분리하고 건조시켜 목적화합물 **14b** (21.3 mg, 42.8 %)를 오일상태로 얻었다.

[0172] ^{1}H-NMR (CDCl$_3$, 500 MHz) : δ 8.23 (1H, d, *J* = 7.8 Hz, Ar-**H**), 7.21 (2H, m, Ar-**H**), 7.05 (1H, t, *J* = 7.3

Hz, Ar-**H**), 5.55(1H, d, *J* = 8.3 Hz, -C**H**OCOCH$_3$), 5.12 (1H, s, -C=C**H**αHβ), 4.93 (1H, s, -C=CHα**H**β), 4.24 (2H, t, *J* = 9.2 Hz, -CH$_2$C**H**$_2$-OH), 3.94 (2H, t, *J* = 9.2Hz, -C**H**$_2$CH$_2$-OH), 3.80 (2H, d, *J* = 7.3 Hz, -N-C**H**$_2$), 3.57 (1H, m, -CO-C**H**(CH$_3$), 3.04 (1H, t, *J* = 6.8 Hz, N-CH$_2$C**H**-C$_2$H$_4$-OH), 2.09 (3H, s, CHOCOC**H**$_3$), 1.56 (2H, m, -CH$_2$C**H**$_2$CH$_2$CH$_2$CH$_3$), 1.45 (4H, m, -CH$_2$CH$_2$C**H**$_2$C**H**$_2$CH$_3$), 1.26 (3H, m, CO(C**H**$_3$)CH), 0.86 (3H, t, *J* = 6.3Hz, -CH$_2$CH$_2$CH$_2$CH$_2$C**H**$_3$).

[0173] **(2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌-1-옥소노난-3-일 아세테이트의 제조(15a)**

[0174] (2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-2-메틸-4-메틸렌-1-옥소노난-3-일 아세테이트 **14a** (344 mg, 0.68 mmol)를 벤젠 (7 ml)에 용해 시킨 후, 2,3-다이클로로-5,6-다이싸이아노-1,4-벤조퀴논 (778.2 mg, 3.42 mmol)을 첨가하여 60℃에서 12시간 동안 교반하였다. 반응이 완결된 것을 확인하고, 에틸아세테이트와 물을 첨가하여 유기층을 분리하였다. 분리한 유기층을 소듐클로라이드 수용액으로 씻어준 후, 무수 황산마그네슘으로 건조시키고, 농축시켰다. 얻어진 잔사를 실리카겔 컬럼크로마토그래피 (n - 헥산 : 에틸아세테이트 = 7 : 1)하여 물질을 분리하고 건조시켜 목적화합물 **15a** (85 mg, 24.8 %)를 오일상태로 얻었다.

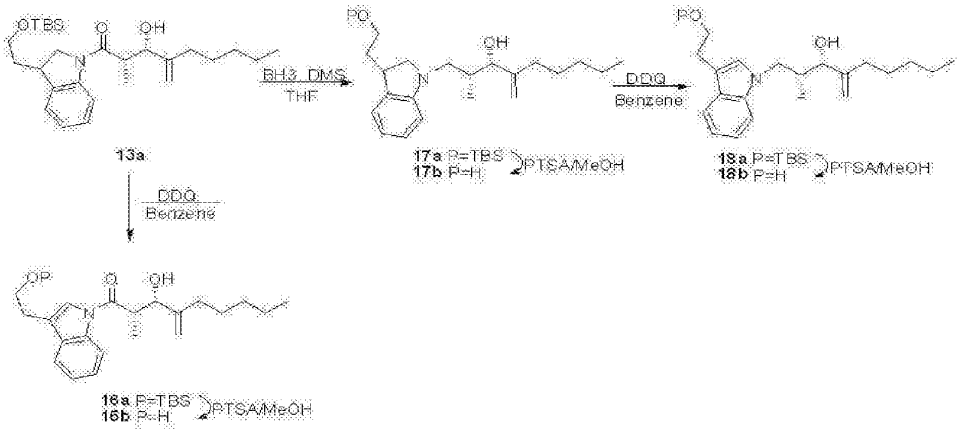
[0175] ^{1}H-NMR (CDCl$_3$, 500 MHz) : δ 8.46 (1H, d, *J* = 8.3Hz, Ar-**H**), 7.72 - 7.70 (1H, m, Ar-**H**), 7.54 - 7.52 (2H, m, Ar-**H**), 7.37-7.27 (1H, m, Ar-**H**), 5.66 (1H, d, *J* = 7.3Hz, -C**H**OCOCH$_3$), 5.12 (1H, s, -C=C**H**αHβ), 4.93 (1H, s, -C=CHα**H**β), 3.92 (2H, m, -CH$_2$-OTBS), 3.53 (1H, m, -CO-C**H**(CH$_3$), 2.93 (2H, m, -CH$_2$CH$_2$-OTBS), 2.11 (3H, s, CHOCOC**H**$_3$), 2.04 (2H, t, *J* = 7.8 Hz, -C**H**$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 1.43 (2H, m, -CH$_2$C**H**$_2$CH$_2$C**H**$_2$CH$_3$), 1.38 (3H, d, *J* = 7.3 Hz, -CO-CH(C**H**$_3$), 1.32 - 1.23 (4H, m, -CH$_2$C**H**$_2$C**H**$_2$CH$_2$CH$_3$), 0.90 (9H, s, -Si-(CH$_3$)$_2$-(C**H**$_3$)$_3$), 0.84 (3H, t, *J* = 7.3 Hz, -CH$_2$CH$_2$CH$_2$CH$_2$C**H**$_3$), 0.04 (6H, d, *J* = 4.89 Hz, -Si-(C**H**$_3$)$_2$-(CH$_3$)$_3$).

[0176] **(2S,3S)-1-(3-(2-하이드록시에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌-1-옥소노난-3-일 아세테이트의 제조(15b)**

[0177] (2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌-1-옥소노난-3-일 아세테이트 **15a** (88.3 mg, 0.17 mmol)을 테트로하이드로퓨란 (5 ml)에 용해 시킨 후, 테트로하이드로퓨란에 1.0 M 로 녹여져 있는 테트라부틸암모니움 플로라이드 (1.76 ml)를 얼음 수조 상태에서 첨가 하였다. 상온에서 2시간 동안 교반한 후, 반응이 완결된 것을 확인하였다. 반응 혼합물에 암모니움 클로라이드 수용액을 넣어 반응을 중지시킨 후, 에틸아세테이트를 넣어 유기층을 분리하였다. 분리한 유기층을 소듐클로라이드 수용액으로 씻어준 후, 무수 황산마그네슘으로 건조시키고, 농축시켰다. 얻어진 잔사를 실리카겔 컬럼크로마토그래피 (n - 헥산 : 에틸아세테이트 = 1 : 1) 하여 물질을 분리하고 건조시켜 목적화합물 **15b** (21.3 mg, 31.3 %)를 오일상태로 얻었다.

[0178] ^{1}H-NMR (CDCl$_3$, 500 MHz) : δ 8.47 (1H, d, *J* = 7.8 Hz, Ar-**H**), 7.55 (1H, d, *J* = 6.84 Hz, Ar-**H**), 7.38 (1H, t, *J* = 7.3 Hz, Ar-**H**), 7.37 (1H, s, Ar-**H**), 7.30(1H, t, *J* = 7.3 Hz, Ar-**H**), 5.60 (1H, d, *J* = 7.3 Hz, -CHOAc), 5.11 (1H, s, -C=C**H**αHβ), 4.94 (1H, s, -C=CHα**H**β), 3.96 (2H, t, *J* = 6.35 Hz, -CH$_2$-OTBS), 3.76 - 3.68 (2H, m, -N-C**H**$_2$), 3.69 (1H, t, *J* = 6.3 Hz, -CH**H**βOH), 3.56 (1H, m, -CO-C**H**(CH$_3$), 2.99 (2H, m, CH$_2$C**H**$_2$OH), 2.09 (3H, s, CHOCOC**H**$_3$), 2.05 (2H, s, C**H**$_2$CH$_2$CH$_2$CH$_2$CH$_3$), 1.42 - 1.52 (6H, m, CH$_2$C**H**$_2$CH$_2$C**H**$_2$CH$_3$), 0.85 (3H, m, -CH$_2$C**H**$_3$).

[0179] **<실시예 10>**



[0180]

[0181] **(2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)-1H-인돌-1-일)-3-하이드록시-2-메틸-4-메틸렌노난-1-온의 제조(16a)**

[0182] (2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-3-하이드록시-2-메틸-4-메틸렌노난-1-온 **13a** (156 mg, 0.31 mmol)를 벤젠 (30 ml)에 용해 시킨 후, 2,3-다이클로로-5,6-다이싸이아노-1,4-벤조큐논 (352.8 mg, 1.55 mmol)을 첨가하여 60℃에서 12시간 동안 교반하였다. 반응이 완결된 것을 확인하고, 에틸아세테이트와 물을 첨가하여 유기층을 분리하였다. 분리한 유기층을 소듐클로라이드 수용액으로 씻어준 후, 무수 황산마그네슘으로 건조시키고, 농축시켰다. 얻어진 잔사를 실리카겔 컬럼크로마토그래피 (n - 헥산 : 에틸아세테이트 = 20 : 1)하여 물질을 분리하고 건조시켜 목적화합물 **16a** (102 mg, 73.9 %)를 오일상태로 얻었다.

[0183] ^{1}H-NMR ($CDCl_3$, 500 MHz) : δ 8.48 (1H, d, *J* = 8.3 Hz, Ar-**H**), 7.55 (1H, d, *J* = 7.8Hz, Ar-**H**), 7.38 (1H, t, *J* = 8.31 Hz, Ar-**H**), 7.33 (1H, s, Ar-**H**), 7.3 (1H, t, *J* = 6.35 Hz, Ar-**H**), 5.28 (1H, s, -C=CH$_\alpha$H$_\beta$), 5.04 (1H, s, -C=CH$_\alpha$H$_\beta$), 4.62 (1H, s, -O**H**), 3.93 (2H, t, *J* = 6.35 Hz, -C**H**$_2$-OTBS), 3.66 (1H, m, -**H**C-OH), 3.31 - 3.27 (1H, m, -CO-C**H**(CH_3)), 2.94 (1H, t, *J* = 6.84 Hz, -CH$_\alpha$H$_\beta$-OTBS), 2.93 (1H, q, *J* = 6.35 Hz, -CH$_\alpha$H$_\beta$-OTBS), 2.07 (1H, m, -C=CH-CH$_\alpha$H$_\beta$-CH_2), 1.97 (1H, m, -C=CH-CH$_\alpha$H$_\beta$-CH_2), 1.50 (2H, m, -CH_2C**H**$_2$$CH_2$$CH_2$$CH_3$), 1.35 (3H, s, -CO-CH(CH3)), 1.34 (4H, m, -$CH_2$$CH_2$$CH_2$$CH_2$$CH_3$), 0.89 (9H, s, -Si-($CH_3$)$_2$-(C**H**$_3$)$_3$), 0.03 (6H, s, -Si-($CH_3$)$_2$-($CH_3$)$_3$).

[0184] **(2S,3S)-3-하이드록시-1-(3-(2-하이드록시에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌노난-1-온의 제조(16b)**

[0185] (2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)-1H-인돌-1-일)-3-하이드록시-2-메틸-4-메틸렌노난-1-온 **16a** (9.9 mg, 0.02 mmol)을 메탄올 (2 ml)에 용해시킨 후, 피리딘 피-톨루엔-썰포네이트를 소량 넣어 준 후 상온에서 2시간 동안 교반하였다. 반응이 완결된 것을 확인한 후, 감압기로 메탄올을 날린 뒤 바로 실리카겔 컬럼크로마토그래피 (n - 헥산 : 에틸아세테이트 = 1 : 1)하여 물질을 분리하고 건조시켜 목적화합물 **16b** (6.9 mg, 94.5 %)를 오일상태로 얻었다.

[0186] ^{1}H-NMR ($CDCl_3$, 500 MHz) : δ 8.49 (1H, d, *J* = 8.3 Hz, Ar-**H**), 7.57 (1H, d, *J* = 7.8 Hz, Ar-**H**), 7.41 - 7.31 (3H, m, Ar-**H**), 5.27 (1H, s, -C=CH$_\alpha$H$_\beta$), 5.04 (1H, s, -C=CH$_\alpha$H$_\beta$), 4.61 (1H, s, C=OCH(CH_3)CH-OH), 3.98 (2H, t, *J* = 5.8 Hz, -C**H**$_2$-OTBS), 3.51 (1H, brs, CH-O**H**), 3.34 - 3.29 (1H, m, C=OCH(CH_3), 3.00 (2H, t, *J* = 6.3 Hz, -CH_2C**H**$_2$-OTBS), 2.12 - 2.06 (1H, m, -C=CH$_\alpha$H$_\beta$), 2.00 - 1.94 (1H, m, -C=CH$_\alpha$H$_\beta$), 1.60 (1H, brs, CH_2C**H**$_2$-O**H**), 1.55 - 1.47 (2H, m, -CH_2C**H**$_2$$CH_2$$CH_2$$CH_3$), 1.36 (3H, d, *J* = 1.46 Hz, -CO-CH(C**H**$_3$)), 1.35 - 1.31 (4H, m, -$CH_2$$CH_2$$CH_2$$CH_2$$CH_3$), 0.90 (3H, t, *J* = 5.8 Hz, CH_2C**H**$_3$).

[0187] **(2R,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-2-메틸-4-메틸렌노난-3-올의 제조(17a)**

[0188] (2S,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-3-하이드록시-2-메틸-4-메틸렌노난-1-온 **13a** (250 mg, 0.54 mmol)를 테트로하이드로퓨란 (15 ml)에 용해 시킨 후, 테트라하이드로퓨란에 2M로 녹여져 있는 보란-다이메틸 썰파이드 (0.5 ml)를 얼음으로 빙냉 시킨 상태에서 첨가하였다. 얼음 수조에서 2시간 동안 교반하여 반응이 완결된 것을 확인한 뒤 메탄올 (1 ml)을 넣어 3시간 동안 더 교반해준다. 반응혼합물을 감압기를 이용하여 날린 후, 바로 실리카겔 컬럼크로마토그래피 (n - 헥산 : 에틸아세테이트 = 15 : 1)하여 물질을 분리하고 건조시켜 목적화합물 **17a** (178 mg, 73.4 %)를 오일상태로 얻었다.

[0189] ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 7.07 (2H, t, *J* = 7.8 Hz, Ar-**H**), 6.66 (1H, t, *J* = 7.3 Hz, Ar-**H**), 6.53 (1H, t, *J* = 6.8 Hz, Ar-**H**), 5.08 (1H, s, -C=CH$_\alpha$H$_\beta$), 4.95 (1H, s, -C=CH$_\alpha$H$_\beta$), 4.25 (1H, d, *J* = 8.3 Hz, -CH(CH_3)C**H**-OH), 3.75 (2H, d, *J* = 5.86 Hz, N-CH_2), 3.64 - 3.54 (1H, m, CH_2CH_2-OTBS), 3.34 (1H, brs, -O**H**), 3.29 (1H, q, *J* = 8.3 Hz, N-CH_2C**H**), 3.16 - 3.07 (1H, m, N-CH$_\alpha$H$_\beta$-CH(CH_3), 3.08 - 2.98 (1H, m, N-CH$_\alpha$H$_\beta$-CH(CH_3), 2.84 (1H, m, CH_2C**H**(CH_3), 2.05 (2H, q, *J* = 7.8 Hz, CH_2CH_2-OTBS), 1.94 (2H, t, *J* = 7.3Hz, =C-CH_2), 1.78 (2H, m, -$CH_2CH_2CH_2$CH_2CH_3), 1.47 (2H, m, -CH_2C$H_2CH_2CH_2CH_3$), 1.30 (2H, m, -CH_2CH_2C$H_2CH_2CH_3$), 0.92 (3H, d, *J* = 7.3 Hz, CH_2CH(CH_3)), 0.92 (9H, s, -Si-(CH_3)$_2$-(CH_3)$_3$), 0.89 (3H, t, *J* = 6.8 Hz, -$CH_2CH_2CH_2CH_2$CH_3), 0.09 (6H, s, -Si-(CH_3)$_2$-(CH_3)$_3$).

[0190] **(2R,3S)-1-(3-(2-하이드록시에틸)인돌린-1-일)-2-메틸-4-메틸렌노난-3-올의 제조(17b)**

[0191] (2R,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-2-메틸-4-메틸렌노난-3-올 **17a** (31 mg, 0.06 mmol)을 메탄올 (1.3 ml)에 용해시킨 후, 피리딘 피-톨루엔-썰포네이트를 소량 넣어 준 후 상온에서 3시간 동안 교반하였다. 반응이 완결 된 것을 확인 한 후, 감압기로 메탄올을 날린 뒤 바로 실리카겔 컬럼크로마토그래피 (n - 헥산 : 에틸아세테이트 = 1 : 1)하여 물질을 분리하고 건조시켜 목적화합물 **17b** (18.4 mg, 79.8 %)를 오일상태로 얻었다.

[0192] ^{1}H-NMR ($CDCl_3$, 500 MHz) : δ7.09 (2H, m, Ar-**H**), 6.70 (1H, t, *J* = 7.3Hz, Ar-**H**), 6.59 (1H, d, *J* = 7.8 Hz, Ar-**H**), 5.09 (1H, s, -C=CH$_\alpha$H$_\beta$), 4.96 (1H, s, -C=CH$_\alpha$H$_\beta$), 4.23 (1H, d, *J* = 7.3 Hz, C**H**(CH_3)CH-OH), 3.77 - 3.71 (2H,m, N -CH_2CH(CH_3)), 3.58 (1H, t, *J* = 8.3 Hz, N-CH$_\alpha$H$_\beta$-CH(CH_3)), 3.52 (1H, t, *J* = 8.8 Hz, N-CH$_\alpha$H$_\beta$-CH(CH_3)), 3.40 (1H, q, *J* = 7.3 Hz, N-CH_2C**H**), 3.20 (2H, m, N-CH_2), 2.92 (1H, m, N-CH_2C**H**(CH_3), 2.05 (2H, m, CH_2CH_2-OH), 1.83 (2H, m, =C-$CH_2CH_2CH_2$CH_2CH_3), 1.47 (2H, m, -$CH_2CH_2CH_2$CH_2CH_3), 1.31 (4H, m, -CH_2C$H_2CH_2CH_2CH_3$), 0.91 (3H, s, -CH_2CH(CH_3), 0.89 (3H, t, *J* = 7.3 Hz, -$CH_2CH_2CH_2CH_2$CH_3).

[0193] **(2R,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌노난-3-올의 제조(18a)**

[0194] (2R,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)인돌린-1-일)-2-메틸-4-메틸렌노난-3-올 **17a** (87.8 mg, 0.19 mmol)를 벤젠 (18 ml)에 용해 시킨 후, 2,3-다이클로로-5,6-다이싸이아노-1,4-벤조큐논 (89.43 mg, 0.39 mmol)을 첨가하여 60℃에서 12시간 동안 교반하였다. 반응이 완결된 것을 확인하고, 에틸아세테이트와 물을 첨가하여 유기층을 분리하였다. 분리한 유기층을 소듐클로라이드 수용액으로 씻어준 후, 무수 황산마그네슘으로 건조시키고, 농축시켰다. 얻어진 잔사를 실리카겔 컬럼크로마토그래피 (n - 헥산 : 에틸아세테이트 = 20 : 1)하여 물질을 분리하고 건조시켜 목적화합물 **18a** (32 mg, 36.6 %)를 오일상태로 얻었다.

[0195] ^{1}H-NMR ($CDCl_3$, 500 MHz) : δ7.59 (1H, d, *J* = 7.8 Hz, Ar-**H**), 7.34 (1H, d, *J* = 7.3 Hz, Ar-**H**), 7.18 (1H, t, *J* = 8.3 Hz, Ar-**H**), 7.08 (1H, t, *J* = 7.8 Hz, Ar-**H**), 6.97 (1H, s, Ar-**H**), 5.05 (1H, s, -C=CH$_\alpha$H$_\beta$),

4.92 (1H, s, -C=CHαHβ), 4.21 (1H, q, J = 8.3 Hz, N-CHαHβCH(CH_3)), 3.99 (1H, q, J = 8.3 Hz, N-CHαHβCH(CH_3)), 3.86 (2H, t, J = 7.3 Hz, CH_2CH_2-OTBS), 3.83 (1H, d, J = 6.3 Hz, CH(CH_3)CH-OH), 2.98 (2H, t, J = 7.3 Hz, CH_2CH_2-OTBS), 2.26 (1H, m, $CH_2CH(CH_3)$), 1.87 (1H, m, =C-CHαHβC_4H_{11}), 1.77 (1H, m, =C-CHαHβC_4H_{11}), 1.30 - 1.16 (6H, m, -$CH_2CH_2CH_2CH_2CH_3$), 0.90 (9H, s, -Si-$(CH_3)_2$-$(CH_3)_3$)), 0.84 (3H, s, $CH_2CH(CH_3)$, 0.83 (3H, t, J = 7.3Hz, -$CH_2CH_2CH_2CH_2CH_3$), 0.04 (6H, s, -Si-$(CH_3)_2$-$(CH_3)_3$)).

[0196] **(2R,3S)-1-(3-(2-하이드록시에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌노난-3-올의 제조(18b)**

[0197] (2R,3S)-1-(3-(2-(터트-부틸디메틸실릴옥시)에틸)-1H-인돌-1-일)-2-메틸-4-메틸렌노난-3-올 **18a** (35.2 mg, 0.08 mmol)을 테트로하이드로퓨란 (4 ml)에 용해 시킨 후, 테트로하이드로퓨란에 1.0 M로 녹여져 있는 테느라부틸암모니움 플로라이드를 얼음 수조 상태에서 첨가하였다. 상온에서 2시간 동안 교반 한 후, 반응이 완결된 것을 확인하였다. 반응 혼합물에 암모니움 클로라이드 수용액을 넣어 반응을 중지시킨 후, 에틸아세테이트를 넣어 유기층을 분리하였다. 분리한 유기층을 소듐클로라이드 수용액으로 씻어준 후, 무수 황산마그네슘으로 건조시키고, 농축시켰다. 얻어진 잔사를 실리카겔 컬럼크로마토그래피 (n - 헥산 : 에틸아세테이트 = 1 : 1)하여 물질을 분리하고 건조시켜 목적화합물 **18b** (12.02 mg, 36 %)를 오일상태로 얻었다.

[0198] ^{1}H-NMR ($CDCl_3$, 500 MHz) : δ7.62 (1H, d, J = 7.8Hz, Ar-**H**), 7.38 (1H, d, J = 8.3 Hz, Ar-**H**), 7.22 (1H, t, J = 8.3 Hz, Ar-**H**), 7.11 (1H, t, J = 7.8 Hz, Ar-**H**), 7.04 (1H, s, Ar-**H**), 5.07 (1H, s, -C=CHαHβ), 4.94 (1H, s, -C=CHαHβ), 4.23 (1H, q, J = 8.3 Hz, N-CHαHβCH(CH_3)), 4.02 (1H, q, J = 7.3Hz, N-CHαHβCH(CH_3)), 3.90 (2H, t, J = 6.3 Hz, CH_2CH_2-OH), 3.83 (1H, s, -CH_2CH(CH_3)CH-OH), 3.04 (2H, t, J = 6.3 Hz, CH_2CH_2-OH), 2.31 - 2.26 (1H, m, -$CH_2CH(CH_3)$), 1.90 (1H, m, =C-CHαHβC_4H_{11}), 1.76 (1H, m, =C-CHαHβC_4H_{11}), 1.60 (1H, brs, -CH(CH_3)CH-**OH**), 1.30 - 1.17 (6H, m, -$CH_2CH_2CH_2CH_2CH_3$), 0.857 (3H, s, $CH_2CH(CH_3)$, 0.84 (3H, s, -$CH_2CH_2CH_2CH_2CH_3$).

[0199] **실험예 1. IL-6 매개성 luciferase 저해활성**

[0200] **<1-1> 형질전환체 제조**

[0201] 96 웰 플레이트에 5×10^4 세포/웰로 HepG2 세포(ATCC HB-8065)를 분주한 후, 10% FBS(v/v), 60.0 mg/ℓ 카나마이신 설페이트(kanamycin sulfate; Gibco., USA) 및 2.0 g/ℓ 탄산수소나트륨($NaHCO_3$; Sigma, USA)이 포함된 DMEM 배양 배지를 사용하여, 37℃에서 5% CO_2의 조건으로 배양접시에 80% 가득찰(confluent) 때까지 배양하였다. 이후 무혈청 배지 50 ㎕로 교환하고, 0.1 ㎍ pSTAT3-TA-Luc (Clontech, CA)와 0.3 ㎕ 리포펙타민 시약(lipofectamin reagent; Invitrogen, USA)의 혼합액을 각 웰에 첨가하여 3시간 반응시킴으로써 pSTAT3-TA-Luc를 형질감염시켰고, 새로 제조한 200 ㎕ DMEM 배양 배지로 바꾸어 추가로 24시간 배양하였다.

[0202] **<1-2> IL-6 및 IL-11 반응성 STAT3 리포터 유전자 검사**

[0203] 상기 형질감염된 세포를 1% BSA/DMEM으로 무혈청 배양(serum starvation)하고 시료를 하기와 같이 1시간 처리한 후 10 ng/mℓ IL-6(R&D system, USA)를 첨가하여 3시간 동안 배양하였다.

[0204] 1: 음성대조군(비처리군);

[0205] 2: 양성대조군(IL-6 10 ng/mℓ);

[0206] 3: 화합물들 (0.3, 1, 3, 10, 30, 100 μM); 및

[0207] 4: Madindoline (0.3, 1, 3, 10, 30, 100 μM)

[0208] 상기 반응한 세포를 PBS로 세척하고 50 ㎕ 용해 완충용액(luciferase assay system, promega, USA)을 넣고 1분간 교반한 후, 30-100 ㎕의 루시퍼라제 기질(luciferase assay system, promega, USA)을 넣고 발색정도를 루미

노미터(luminometer; EG&G BERTHOLD, USA)로 5분 안에 측정하였다.

[0209] 각 화합물들의 IL-6 유도 루시퍼라제 저해활성 효과의 IC_{50} 값은 도 1과 같으며 화합물 **4d** 는 농도의존적으로 IL-11 유도 루시퍼라제 저해활성을 도 2와 같이 나타냈으며 그 IC_{50} 값은 2.1 mM이었다.

[0210] **<1-3> IL-6에 의해 유도된 STAT3 인산화 저해활성 검정**

[0211] 6 웰 플레이트에 5×10^4 세포/웰로 HepG2 세포를 분주하여 배양접시에 80% 가득차게 배양한 후, 무혈청 배지로 교환하여 추가로 6시간 배양하고 하기와 같이 시료를 30분간 처리하였다.

[0212] 1: 음성대조군(비처리군);

[0213] 2: 양성대조군(IL-6 20 ng/mℓ); 및

[0214] 3: 화합물 4d 처리군(1, 3, 0.39, 10, 30 및 100 μM)

[0215] 이후 20 ng/mℓ IL-6를 처리하여 10분간 반응한 뒤 40 ㎕ 용해 완충용액[pH 8, 20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM PMSF, 20 mM 류펩틴(leupeptin), 20 ㎍/mℓ 아포로토닌(aprotonin); Sigma, USA]을 사용하여 세포를 용해시킨 후, 원심분리 (13000g, 15분)하여 단백질이 녹아있는 상등액을 수득하였다. 이때, 시료 및 IL-6을 처리하지 않은 HepG2 세포를 대조군으로 사용하였다. 단백질의 농도는 DC 단백질 검사 키트(Bio-Rad, USA)를 이용하여 정량하였고, 10% SDS 폴리아크릴아마이드 겔(SDS-PAGE)에 단백질을 로딩하여 30 mA에서 2시간 동안 전기영동 하였다. 전기영동이 끝난 후 겔의 단백질을 PVDF 멤브레인(Westran S, pore size 0.2 ㎛; Whatman, USA)으로 90 V에서 90분 동안 전사시켰다. 전사된 멤브레인을 Tris-완충용액(T-TBS; 50 mM Tri-HCl, pH 7.6, 150 mM NaCl, 0.2 % Tween-20, 5% skim milk; Sigma, USA)으로 4℃에서 12시간 차단하고 T-TBS로 5번 세척하였다. 상기 멤브레인에 일차항체로 phospho-STAT3(1:1000 희석)의 다중클론 항체를 2시간 동안 처리하였다. T-TBS로 5번 세척 후 이차항체로 HRP-결합 항-래빗 항체(1:5000 희석)를 1시간 반응시켰다. T-TBS로 세척한 다음 암실에서 ECL 키트(Amersham, USA)를 이용하여 필름을 현상시켰다.

[0216] 그 결과, 도 3에서 나타난 바와 같이 본 발명의 화학식 1 화합물은 IL-6 유도 STAT3 인산화 저해 활성을 나타내었다.

[0217] **<1-4> IL-6에 의해 유도된JAK2와 gp130 인산화 저해활성**

[0218] 6 웰 플레이트에 5×10^4 세포/웰로 HepG2 세포를 분주하여 배양접시에 80% 가득차게 배양한 후, 무혈청 배지로 교환하여 추가로 6시간 배양하고 하기와 같이 시료를 30분간 처리하였다.

[0219] 1: 음성대조군(비처리군);

[0220] 2: 양성대조군(IL-6 20 ng/mℓ); 및

[0221] 3: 화합물 **4d** 처리군(1, 3, 0.39, 10, 30 및 100 μM)

[0222] 이후 20 ng/mℓ IL-6를 처리하여 10분간 반응한 뒤 40 ㎕ 용해 완충용액[pH 8, 20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM PMSF, 20 mM 류펩틴(leupeptin), 20 ㎍/mℓ 아포로토닌(aprotonin); Sigma, USA]을 사용하여 세포를 용해시킨 후, 원심분리 (13000g, 15분)하여 단백질이 녹아있는 상등액을 수득하였다. 여기에 항-JAK2 항체와 anti-gp130항체(Cell signaling, INC.)를 사용하여 4℃에서 12시간 반응시킨 후, protein A/G plus agarose (Santa Cruz Biotechnology, USA)로 침강시켰다. 원심분리 후 침강물은 완충용액으로 3회 세척 후 전기영동 시료로 사용하였다. 이 때 IL-6 및 시료를 처리하지 않은 HepG2 세포를 대조군으로 사용하였다. 단백질의 농도는 Bio-Rad DC protein assay kit를 이용하여 정량하였고, 8 % SDS-polyacryl-amide gels (SDS-PAGE)에서 단백질을 loading하여 30 mA에서 2시간동안 전기영동 하였다. 전기영동이 끝난 후 gel의 단백질을 PVDF membrane (WeatranS, pore size 0.2 ㎛)으로 90V에서 90분 동안 전사시켰다. 전사된 membrane을 Tris-buffered solution (T-TBS; 50 mM Tri-HCl, pH 7.6, 150 mM NaCl, 0.2 % Tween-20, 5% skim milk)로 4℃에서 12시간 blocking하고 T-TBS로 5번 세척하였다. 이 membranes에 일차항체로

phospho-tyrosine antibody (1:1000 dilutions, respectively)의 polyclonal antibodies를 2시간 동안 처리하였다. T-TBS로 5번 세척 후 이차항체로 HRP-conjucated anti-mouse antibody (1:5000 dilutions)를 1시간 반응시켰다. T-TBS로 세척한 다음 암실에서 ECL을 이용하여 필름을 현상시켰다. 그 결과, 도 4에서 나타난 바와 같이 본 발명의 화학식 1 화합물은 IL-6 유도 JAK2 및 gp130의 인산화 저해 활성을 나타내었다.

[0223] **<1-5> Insulin에 의해 유도된 adipogenesis에 대한 화합물 4d의 효과**

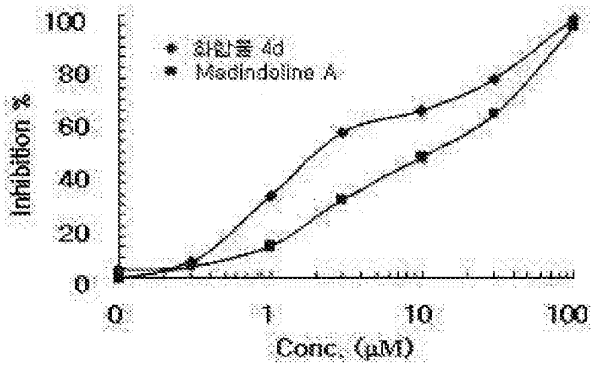
[0224] 3T3L1 세포를 24 웰 세포 배양 플레이트에 confluence하게 되기 이틀 전에 분화배지(containing 5mg/ml insulin, 1mM dexamethasone, and 0.5 mM IBMX)로 바꾸어주고 8일 동안 2일에 한번 씩 분화배지로 바꾸어주면서 실험을 하였다. 이때 분화용 배지를 사용하지 않거나 분화배지에 IL-6 나 화학식 1 각각 단독으로 혼합하여 처리한 세포 그리고 분화배지에 IL-6와 화학식 1을 함께 혼합하여 처리한 세포를 비교하여 실험하였다. 8일후 세포는 PBS로 세척하고 PBS에 3.7% formaldehyde가 첨가된 용액으로 고정을 시킨 후, Oil Red O dye를 처리하여 1 시간동안 염색을 한다. 그리고 25 % isopropanol을 첨가하여 세척하고 현미경으로 관찰하였다. 그 결과, 도 5에 나타난 바와 같이, 본 발명의 화학식 1의 화합물은 인슐린-유도 아디포제네시스(adipogenesis)를 저해하는 IL-6 효과를 회복하는 활성을 나타내었다.

도면

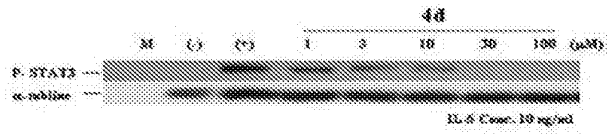
도면1

Compound No.	IC_{50} (μM)
4a	>100
4b	20.1
4c	27.0
4d	5.9
4e	7.5
4f	>100
4g	11.7
5	37.1
7a	26.1
7b	35.9
9	35.2
13a	40.0
13b	16.5
14a	>100
14b	>100
15a	>100
15b	>100
16a	58.6
16b	7.5
17a	48.1
17b	>100
18a	62.6
18b	4.4
Madindoline A	21.0

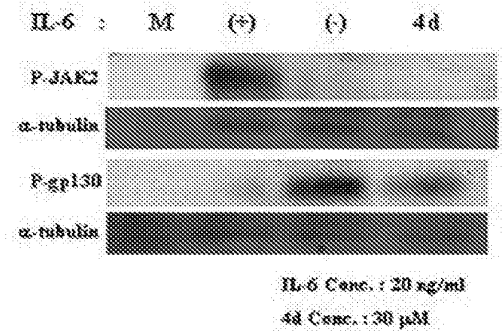
도면2



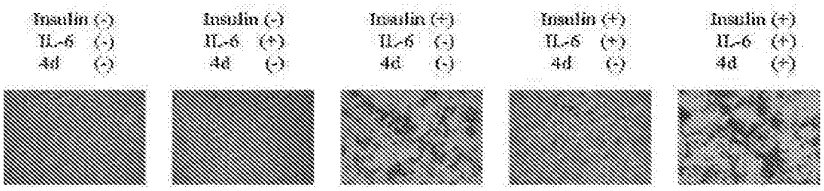
도면3



도면4



도면5



Espacenet

Bibliographic data: KR20110047179 (A) — 2011-05-06

New oxazolidin and indole compounds, process for the preparation thereof and pharmaceutical composition comprising the same

Inventor(s):

Applicant(s):

Classification:	**- international:** ***A61K31/404; A61P29/00; C07D209/26***
	- cooperative:
Application number:	KR20110035774 20110418 Global Dossier
Priority number(s):	KR20110035774 20110418
Also published as:	KR101133769 (B1)

Abstract of KR20110047179 (A)

PURPOSE: A pharmaceutical composition containing novel oxazolidine and indole compound, or pharmaceutically acceptable salt is provided to suppress IL-6 or IL-11 signal transduction system and to be used as an anticancer agent and anti-inflammatory agent. CONSTITUTION: An indole compound is denoted by chemical formula 1. A pharmaceutical composition for preventing and treating inflammatory diseases or cancer contains 0.0001-10 weight% of indole compound of chemical formula 1 or pharmaceutically acceptable salt. The composition is manufactured in the formulation of powders, granules, tablets, capsules, suspensions, emulsions, syrups, suppository, and excipient. The pharmaceutical composition is administered by oral, rectal or venous, muscular, subcutaneous, or intracerebroventricular injection.

Notice

DESCRIPTION KR20110047179

TECHNICAL FIELD The present invention relates to a novel oxazolidine-based compound and an indole-based compound, a process for preparing the same, and a pharmaceutical composition containing the same.

[0001]
The present invention relates to novel anti-inflammatory and anticancer agents containing synthetic compounds, oxazolidine-based compounds and indole-based compounds, a method for their synthesis, or a pharmaceutically acceptable salt thereof as an active ingredient.

[0002]

Interleukin-6 (IL-6) is a cytokine, also called B cell stimulating factor 2 (BSF2) or interferon beta2 (INF-beta2).

IL-6 has been found as a differentiator involved in the activation of B lymphocytes (Hirano, T. et al., Nature (1986) 324, 73-76). It has since been shown to be a multifunctional cytokine that affects various cell functions (Akira, S. et al., Adv. In Immunology (1993) 54, 1-78). IL-6 transports its biological activity via two types of proteins on the cell membrane. One is the IL-6 receptor, a protein that IL-6 binds. The IL-6 receptor is a membrane-bound protein with a molecular weight of approximately 80 kD that is expressed through the cell membrane. And the other is a membrane

protein gp130 having a molecular weight of about 130 kD, which belongs to the signal transduction of non-ligand binding. IL-6 and IL-6 receptors form an IL-6 / IL-6 receptor complex, which in turn binds to gp130. (Taga et al., J. Exp. Med. (1987) 166, 967). After binding of ligands and receptors, Janus Kinases 2 (JAK2) is activated in the cells by transphosphorylation. Activated JAK2 phosphorylates several tyrosine residues of the cytoplasmic domains and this translates into STAT3 signal transducers and activators of transcription with SH2 or other phosphorylated tyrosine binding motifs 3), which serves as a docking site for proteins in the cytoplasm. STAT3 bound to the cytoplasmic domain of the receptor is phosphorylated by JAK2 and then released from the receptor. The activated STAT3 binds to each other in the cytoplasm to form a homo- or hetero-dimer, enters the nucleus, binds to the recognition sequence of the target gene, and increases transcription (Levy, DE, et al., Nat Rev Mol Cell Biol, 2002, 3, 651-62, Darnell, JE, Jr., Science, 1997, 277, 1630-1635).

[0003]

Such IL-6-mediated signaling pathways have been reported to be associated with inflammatory diseases and various cancers, and thus inhibition of the IL-6-mediated signaling pathway is therapeutically useful. Currently, anti-IL-6 R antibodies are the most studied for inhibitory function of IL-6 signal transduction system. This anti-IL-6 R antibody has been reported to inhibit synovial cell growth against rheumatoid arthritis (International Patent Publication No. 98/11020), and has been shown to be effective in the treatment of proliferative diseases such as transgene hypermethylation, hyperimmunoglobulinemia, anemia, nephritis, cachexia, rheumatoid arthritis, (See WO 96/12503), in the treatment of diseases that contribute to IL-6 products such as cattle disease, cattle disease, and angina pectoris. It has also been described in the prophylactic / protective agent for susceptible T cell related diseases such as multiple sclerosis, uveitis, chronic thyroiditis, delayed hypersensitivity, contact dermatitis and atopic dermatitis (WO 98/42377) (International Patent Publication No. 98/42377). In addition, in a report describing a therapeutic agent for Crohn's disease (International Patent Publication No. 99/47170), its active ingredient was an anti-IL-6R antibody. Patents describing therapeutic agents for pancreatitis have also been reported as active ingredients (WO 00/10607) and in International Patent Publication No. 02/3492, which describes a therapeutic agent for psoriasis, the active ingredient is anti-IL-6R Lt; / RTI & gt; In addition, in WO 02/080969, which describes a therapeutic agent for inflammatory idiopathic atrophy, its active ingredient is an anti-IL-6R antibody. However, these proteins may have epitopes that can be recognized as foreign proteins and may still be immunogenic when used as therapeutic agents. However, small molecule compounds that are not proteins have not been recognized by these immune

systems, and many studies have been made.

[0004]

In addition, the IL-6 signaling pathway associated with cancer is much related to its intermediate mediator, STAT3. It has been reported to be involved in various types of cancer, including myeloma, breast, prostate, brain, head and neck carcinoma, melanoma, leukemia and lymphoma, especially chronic myelogenous leukemia and multiple myeloma (Niu et al., Cancer Res. , 1999, 59, 5059-5063). Cells derived from both mouse and human prostate cancer have been found to have structurally activated STAT3, and STAT3 has been shown to have some acute leukemia (Gouilleux-Gruart, V. Back, Leuk. Lymphoma, 1997, 28, 83-88) and T cell lymphoma (Yu, C.L. Et al., J. Immunol., 1997, 159, 5206-5210). Interestingly, STAT3 has been shown to be structurally phosphorylated on serine residues in chronic lymphocytic leukemia (Frank, D. A., et al., J. Clin. Invest., 1997,100, 3140-3148). STAT3 has been found to be structurally active in myeloma tumor cells, both in bone marrow mononuclear cells and in cultures from patients with multiple myeloma. These cells are resistant to Fas-mediated cell death and express high levels of Bcl-xL. STAT3 signaling has been shown to be essential for the survival of myeloma tumor cells by conferring resistance to apoptosis (Catlett-Falcone, R. Immunity, 1999, 10, 105-115). In recent years, IL-6 is secreted and IL-6 is eliminated ideally in cancer patients induced by Ras, including pancreatic cancer. In addition, tumor growth and angiogenesis caused by Ras are suppressed and tumor size is reduced (Brooke Ancrile et al., Gene & Development, 2007, 21, 1714-1719). In addition, IL-6-induced gp130 / JAK / STAT3 pathway is emerging as a new target for chemotherapy, as STAT3 is activated by IL-6 overexpression in EGFR mutated lung adenocarcinoma (Sizhi Paul Gao et al., J. Clin.

Invest. 2007, 117, 38463856).

[0005]

Interleukin-11 (IL-11), an inflammatory cytokine belonging to the IL-6 family, has almost the same signal transduction system as IL-6. Its expression is increased in hematopoiesis, (Jackson CB et al. J Pathol 2007, 213, 140-151). Recently, it has been reported that IL-11 binds to its receptor, IL-11R.alpha., And gp130 to promote gastric cancer, colon cancer cell proliferation and cancer invasion (Nakayama T et al., 2007, 30,

825-833, Ernst et al. Have shown that the smad7 is activated by the IL-11 / Stat3 signal and the smad activator that induces the TGFβ signal is blocked at the same time. Therefore, the oncogenic program (antiapoptotic gene, proangiogenic gene, proliferative gene) has been activated to induce inflammatory gastric tumors (Ernst et al., J. Clin. Invest 2008, 118 (5), 1728-1738). Therefore, the gp130 / JAK / STAT3 pathway by IL-11 is emerging as a new target for chemotherapy.

[0006]
Thus, the present inventors have searched IL-6 and IL-11-induced inhibitors of the signal transduction system from organic synthesis samples and found that the newly synthesized oxazolidine-based and indole-based compounds inhibit IL-6 and IL-11 And thus it is useful as an agent for the treatment of inflammatory diseases and cancer, thereby completing the present invention.

One object of the present invention is to provide a novel oxazolidine-based, indole-based compound, or a pharmaceutically acceptable salt thereof.

It is another object of the present invention to provide a pharmaceutical composition for the prophylaxis and treatment of inflammatory diseases or cancer, which comprises the novel compound or a pharmaceutically acceptable salt thereof.

Hereinafter, the present invention will be described in detail. In one aspect, the present invention relates to an oxazolidine-based compound represented by the following general formula (1): or a pharmaceutically acceptable salt thereof. Wherein R & lt; 1 & gt; is hydrogen or straight chain, branched chain or cycloalkyl of C1-10, or substituted or unsubstituted benzyl; R2 is hydrogen, or straight chain, branched or cycloalkyl of C1-10; R3 is hydrogen, or straight chain, branched chain or cycloalkyl of C1-10, or substituted or unsubstituted benzyl; R4 is hydrogen, or a straight chain, branched chain or cycloalkyl of C1-10, or substituted or unsubstituted benzyl, or R3 and R4 together form cyclohexene. More preferably, R1 is isopropyl or benzyl; R2 is C1-5 straight chain, branched or cycloalkyl; R3 is hydrogen; And R4 is C1, C4-7 alkyl, isopropyl, or benzyl, or R3 and R4 together form cyclohexene. Preferred examples of the compound of the formula (1) of the present invention are as follows: 1) (S) -3 - ((2S, 3S) -3-hydroxy-2,4-dimethylpent- 4-isopropyloxazolidin-2-one (4a), 2) (S) -3 - ((2S, 3S) -3-hydroxy- (4S) -3 - ((2S, 3S) -3-hydroxy-2-methyl-4-methyleneoctanoyl) -4-isopropyloxazoli (4S) -3-hydroxy-2-methyl-4-methylenenanoyl) -4-isopropyloxazolidin-2 -one (4d), 5) (S) -3 - ((2S, 3S) -3-

Hydroxy-2- methyl-4-methylenedecanoyl) -4- isopropyloxazolidin- Methyl-4-methylene undecanoyl) -4-isopropyloxazolidin-2-one (4f), 7 (7S) -3-hydroxy- (4 g), 8 & lt; RTI ID = 0.0 & gt; (3-hydroxy-) (S) -3 - ((2S, 3S) -3-cyclohexenyl-3-hydroxy-2-methylpropanoyl) -4- 2-one (4h), 9) (R) -3 - ((2R, 3R) -3-hydroxy-2- methyl-4-methylenonanoyl) -4-isopropyloxazoli Methyl-4-methylenonanoyl) oxazolidin-2-one (5), 10) Methyl-4-methylenonanoyl) oxazolidin-2-one (7b), 11) (S) , And 12) (S) -3 - ((2S, 3R) -3-hydroxy-2-methylnonanoyl) -4-isopropyloxazolidin-2-one (9).

Compounds R1R442'3'4aCH (CH3) 2CH3SSS4bCH (CH3) 2CH (CH3) 2SSS4cCH (CH3) 2C4H9SSS4dCH (CH3) 2C5H11SSS4eCH (CH3) 2C6H13SSS4fCH (CH3) 2C7H15SSS4gCH (CH3) 2benzylSSS4hCH (CH3) 21-CyclohexeneSSS5CH) 2RR7abenzylC5H11SSS7bbenzylC5H11SRS9CH (CH3) 2C6H15SSR The oxazolidine compound of the formula (1) of the present invention can be used in the form of a pharmaceutically acceptable salt, and includes all salts, hydrates and solvates prepared by a conventional method. Salts are useful as acid addition salts formed by pharmaceutically acceptable free acids. As the free acid, inorganic and organic acids can be used. As the inorganic acid, hydrochloric acid, bromic acid, sulfuric acid, phosphoric acid and the like can be used. As the organic acid, citric acid, acetic acid, lactic acid, tartaric acid, fumaric acid, formic acid, Sulfonic acid, 4-nitrobenzenesulfonic acid, 4-toluenesulfonic acid, 4-toluenesulfonic acid, 4-nitrobenzenesulfonic acid, benzenesulfonic acid, maleic acid, benzoic acid, gluconic acid, glycolic acid, Glutaric acid, embossic acid, glutamic acid, and aspartic acid. In another aspect, the present invention relates to an indole compound represented by the following formula (2) or a pharmaceutically acceptable salt thereof. Wherein R & lt; 1 & gt; is hydrogen or substituted or unsubstituted C1-10 alkyl; R2 is hydrogen, or straight chain, branched or cycloalkyl of C1-10; R3 is hydrogen, or straight chain, branched chain or cycloalkyl of C1-10, or substituted or unsubstituted benzyl; R4 is hydrogen, or straight chain, branched chain or cycloalkyl of C1-10, or substituted or unsubstituted benzyl; X is hydrogen, halogen, hydroxy, methoxy, substituted or unsubstituted C1-10 alkyl; Y is hydrogen or oxygen; Z is hydroxy, C1-10 alkoxy, -OCOCH3 or oxygen; The above is a single or double bond.

More preferably, R1 is hydroxyethyl or tert-butyldimethylsilyloxyethyl; R2 is C1-5 straight chain, branched or cycloalkyl; R3 is hydrogen; R4 is hydrogen or straight chain, branched or cyclic alkyl of C1-5; X is hydrogen, halogen, hydroxy, methoxy, substituted or unsubstituted alkyl; Y is hydrogen or oxygen; Z is hydroxy, C1-5 alkoxy, -OCOCH3 or oxygen; And the above is a single or double bond. (2S, 3S) -1- (3- (2- (tert-butyldimethylsilyloxy) ethyl) indolin-1-yl) (13a), 2) (2S, 3S) -1- (3- (2- (tert-

butyldimethylsilyloxy) ethyl) indoline (2S, 3S) -1- (3- (2- (tert-butyldimethylsilyl) -1- 4-methylene-1-oxonan-3-yl acetate (15a), 4) (2S, 3S) -1- (15b), 5) (2S, 3S) -1- (3-methoxybenzyl) -1,3-dihydro- Methyl-4-methylenenonan-1-one (16a), 6) (2S, (16b), 7) (2R (3R) -3-hydroxy-1- , 3S) -1- (3- (2- (tert-butyldimethylsilyloxy) ethyl) indolin-1-yl) -2-methyl- 3-ol (17a), 8) (2R, 3S) -1- (3- (2- (tert- butyldimethylsilyloxy) ethyl) (2R, 3S) -1- (3- (2-hydroxyethyl) -1H-indol-1-yl) -2-methyl- (18b), 10) (2S, 3S) -3-Hydroxy-1- (3- (2-hydroxyethyl) indolin- (13b), 11) (2S, 3S) -1- (3- (2-Hydroxyethyl) indolin-1-yl) -2- methyl- Acetate (14b) and 12) (2R, 3S) -1- (3- (2-Hydroxyethyl) indolin-1-yl) -2-methyl-4-methylenenan-3-ol (17b).

Compound R 1 YZ Indole derivative bond 13a (CH2) 2-OTBSOH Single bond 14a (CH2) 2-OTBSOOCOCH3 Single bond 15a (CH2) 2-OTBSOOCOCH3 Double bond 15b (CH2) 2-OHOOCOCH3 Double bond 16a (CH 2) 2 -OHHH double bond 13b (CH 2) 2 -OHOH double bond 17a (CH 2) 2-OTBSHH single bond 18a (CH 2) -OHOOCOCH3 single bond 17b (CH2) 2-OHHH single bond The indole compound of formula (2) of the present invention can be used in the form of a pharmaceutically acceptable salt, and all salts, hydrates and solvates . Salts are useful as acid addition salts formed by pharmaceutically acceptable free acids. As the free acid, inorganic and organic acids can be used. As the inorganic acid, hydrochloric acid, bromic acid, sulfuric acid, phosphoric acid and the like can be used. As the organic acid, citric acid, acetic acid, lactic acid, tartaric acid, fumaric acid, formic acid, Sulfonic acid, 4-nitrobenzenesulfonic acid, 4-toluenesulfonic acid, 4-toluenesulfonic acid, 4-nitrobenzenesulfonic acid, benzenesulfonic acid, maleic acid, benzoic acid, gluconic acid, glycolic acid, Glutaric acid, embossic acid, glutamic acid, and aspartic acid. Reaction Scheme 1 Reaction Scheme 3 Reaction Scheme 4 Reaction Scheme 6 Reaction Scheme 7 Reaction Scheme 9 Reaction Scheme 10 The above reactions or all the reactions after completion of the reaction The product can be isolated and purified by conventional post treatment methods such as chromatography, recrystallization and the like. The isopropyloxazolidine compound of the present invention represented by the formula (1) and the indole compound represented by the formula (2) can be synthesized by a synthetic method and can be obtained by any conventional method, and commercially available reagents can be used. In another aspect, the present invention relates to a pharmaceutical composition for the prophylaxis and treatment of inflammatory diseases or cancer, which comprises the compound of formula (I) or (I) or a pharmaceutically acceptable salt thereof.

The compounds according to the present invention are excellent in the effect of inhibiting the signal transduction system induced by IL-6 or IL-11, and thus are useful for the treatment of diseases mediated by IL-6 or IL-11, in particular inflammatory

diseases or cancer diseases There is an excellent effect on prevention. Wherein said inflammatory disease is selected from the group consisting of rheumatoid arthritis, osteoporosis, transglottial hyperplasia, hyperimmunoglobulinemia, anemia, nephritis, cachexia, climacteric disease, angiostatic nephritis, multiple sclerosis, uveitis, chronic thyroiditis, delayed hypersensitivity, contact dermatitis atopic dermatitis, Including but not limited to all inflammatory diseases mediated by IL-6 or IL-11, including, but not limited to, acanthosis, Crohn's disease, pancreatitis, psoriasis, burning idiopathic atrophy, diabetes and Alzheimer's. The cancer diseases may be selected from the group consisting of pancreatic cancer, breast cancer, prostate cancer, brain tumor, head and neck carcinoma, melanoma, myeloma, melanoma, leukemia, lymphoma, liver cancer, gastric cancer, colon cancer, ovarian cancer, ovarian cancer, But are not limited to, cancer of the anus, colon cancer, fallopian tube carcinoma, endometrial carcinoma, cervical carcinoma, vaginal carcinoma, vulvar carcinoma, Hodgkin's disease, bladder carcinoma, renal carcinoma, ureter carcinoma, renal pelvic carcinoma, But are not limited to, all cancer diseases mediated by IL-6 or IL-11. The pharmaceutical composition of the present invention may contain 0.0001 to 10% by weight, preferably 0.001 to 1% by weight of the above compound, based on the total weight of the composition. In addition, the composition comprising the oxazolidine compound of the present invention and the indole compound (Formula 2) may further include an appropriate carrier, excipient, and diluent commonly used in the production of a pharmaceutical composition. The pharmaceutical dosage form of the oxazolidine compound of the present invention represented by the formula (1) and the indole compound of the formula (2) may be used in the form of a pharmaceutically acceptable salt thereof, and may be used alone or in combination with another pharmacologically active compound It can also be used as an appropriate set. The composition comprising the oxazolidine compound according to the present invention and the compound represented by the formula (2), which is an indole compound, can be administered orally or parenterally in the form of powders, granules, tablets, capsules, suspensions, emulsions, syrups, Formulation, external preparation, suppository, and sterile injection solution.

Examples of carriers, excipients and diluents that can be included in the composition containing the extract include lactose, dextrose, sucrose, sorbitol, mannitol, xylitol, erythritol, maltitol, starch, acacia rubber, alginate, gelatin, calcium phosphate, calcium silicate , Cellulose, methylcellulose, microcrystalline cellulose, polyvinylpyrrolidone, water, methylhydroxybenzoate, propylhydroxybenzoate, talc, magnesium stearate and mineral oil. In the case of formulation, a diluent or excipient such as a commonly used filler, an extender, a binder, a wetting agent, a disintegrant, a surfactant or the like is used. Solid formulations for oral administration include tablets, pills, powders, granules, capsules and the like, which may contain at least one excipient such as starch, calcium carbonate, sucrose, (sucrose), lactose, gelatin and the like. In addition to simple

excipients, lubricants such as magnesium stearate and talc are also used. Liquid preparations for oral use may include various excipients such as wetting agents, sweeteners, fragrances, preservatives, etc. in addition to water and liquid paraffin, which are simple diluents commonly used in suspension, liquid solutions, emulsions and syrups have. Formulations for parenteral administration include sterile aqueous solutions, non-aqueous solutions, suspensions, emulsions, freeze-dried preparations, and suppositories. Examples of the suspending agent include propylene glycol, polyethylene glycol, vegetable oil such as olive oil, injectable ester such as ethyl oleate, and the like. As the base of suppositories, witepsol, macrogol, tween 61, cacao paper, laurin, glycerogelatin and the like can be used. The preferable dose of the oxazolidine compound of the present invention represented by formula (1) and the indole compound of formula (2) varies depending on the condition and body weight of the patient, degree of disease, drug form, route of administration and period of time, Can be.

However, for the desired effect, the extract or the compound of the present invention is preferably administered at 0.0001 to 100 mg / kg, preferably 0.001 to 100 mg / kg per day. The administration may be carried out once a day or divided into several times. In addition, the pharmaceutical composition of the present invention can be administered to mammals such as rats, mice, livestock, and humans in various routes. All modes of administration may be expected, for example, by oral, rectal or intravenous, intramuscular, subcutaneous, intra-uterine or intracerebroventricular injections.

INDUSTRIAL APPLICABILITY As described above, the present invention relates to an oxazolidine-based compound or an indole-based compound represented by the formula (I) or a pharmaceutically acceptable salt thereof, which has the inhibitory activity of a signal transduction system induced by IL-6 and IL- Or a composition comprising the same, wherein the composition is used for the treatment and / or prophylaxis of rheumatoid arthritis, osteoporosis, transglottial hyperplasia, hyperimmunoglobulinemia, anemia, nephritis, cachexia, cattle disease, angioplasty nephritis, multiple sclerosis, uveitis, chronic thyroiditis, Inflammatory diseases including atopic dermatitis, systemic lupus erythematosus, Crohn's disease, pancreatitis, psoriasis, inflammatory idiopathic diabetes mellitus, diabetes mellitus and Alzheimer's disease and inflammatory diseases including pancreatic cancer, breast cancer, prostate cancer, brain tumor, head and neck carcinoma, melanoma, myeloma, Lymphoma, liver cancer, stomach cancer, colon cancer, bone cancer, uterine cancer, ovarian cancer, rectal cancer, esophageal cancer, small intestine cancer, A medicament for the prophylaxis and treatment of cancers including endometrial carcinoma, cervical carcinoma, vaginal carcinoma, vulvar carcinoma, Hodgkin's disease, bladder cancer, kidney cancer, ureter cancer, kidney cell carcinoma, renal pelvic carcinoma and central nervous system tumor Can be usefully used.

[0082] BRIEF DESCRIPTION OF THE DRAWINGS FIG. 1 is a graph showing the IC50 of the inhibitory activity of luciferase induced by isopropyloxazolidine-based compound and indole-based compound to IL-6 in HepG2 cells. FIG. 2 is a graph showing the inhibitory activity of luciferase, which is induced by IL-11 in HepG2 cells, according to the isopropyloxazolidine-based compound represented by Chemical Formula 4d according to the present invention. FIG. 3 is a graph showing IL-6-induced STAT3 phosphorylation-inhibiting activity in HepG2 cells of the isopropyloxazolidine-based compound represented by Chemical Formula 4d according to the present invention. FIG. 4 is a graph showing IL-6-induced JAK2 and gp130 phosphorylation-inhibiting activity in U266 cells according to the present invention, wherein the isopropyloxazolidine-based compound represented by Chemical Formula 4d. FIG. 5 is a graph showing the activity of the isopropyloxazolidine-based compound represented by Chemical Formula 4d according to the present invention to restore the IL-6 effect inhibiting insulin-induced adipogenesis in 3T3L1 cells.

[0083] Hereinafter, preferred embodiments of the present invention will be described in order to facilitate understanding of the present invention. However, the following examples are provided only for the purpose of easier understanding of the present invention, and the present invention is not limited by the examples.

[0084] EXAMPLES Synthesis of Compounds Represented by Formulas (1) and (2)

[0085] & Lt; Example 1 & gt;

[0087] 3Methyl-2-methylenebutanal & lt; / RTI & gt; (2b)

[0088] 1 (440 mg, 50 mmol, 0.93 eq) and a dimethylamine hydrochloride solution (0.434 ml, 58 mmol, 1 eq, 37%) and an aqueous solution of dimethylamine hydrochloride were added at 70 [deg.] C to 500 mg (58 mmol, 1 eq) C for 48 hours. The completion of the reaction was confirmed by TLC. After confirming that all the compounds became 2b, water was added to quench the reaction, and diethylether was added to separate the organic layer. The organic layer was dried over anhydrous magnesium sulfate and concentrated to give yellow liquid material 2b (44-62%). The reaction was immediately stirred without any column.

[0089] 1(1H, s, -C = CH? H?), 5.94 (1H, s, -C = CH? H?), 2.78 -CH (CH3) 2), 1.07 (6H, d, J = 6.8Hz, -CH (CH3)

[0090] 2- Preparation of methylene hexanal (2c)

[0091] Using the same method as above, 2c (44-62%) of yellow liquid material was obtained.

[0092] 1(1H, s, -C = CHαHβ), 5.98 (1H, s, -C CH.alpha.H.beta.), 2.23 (2H, t, J = 7.3 Hz, -CH2CH2CH2CH3), 1.35-1.30 (4H, m, -CH2CH2CH2CH3), 0.90 (3H, t, J = 7.3Hz, -CH2CH2CH2CH3)

[0093] 2- & lt; / RTI & gt; methyleneheptanal (2d)

[0094] Using the same method as above, 2d (44-62%) of yellow liquid material was obtained.

[0095] 1(1H, s, -C = CHαHβ), 5.97 (1H, s, -C = CHαHβ), 2.21 (2H, t, J = 7.8Hz, -CH2CH2CH2CH2CH3), 1.31-1.25 (6H, m, -CH2CH2CH2CH2CH3), 0.87 (3H, t, J = 6.8Hz, -CH2CH2CH2CH2CH3)

[0096] 2- & lt; / RTI & gt; methylene octanal (2e)

[0097] Using the same method as above, 2e (65%) of yellow liquid material was obtained.

[0098] 1(1H, s, -C = CHαHβ), 5.97 (1H, s, -C = CHαHβ), 2.21 (2H, t, J = 7.8Hz, -CH2CH2CH2CH2CH2CH3), 1.31-1.25 (8H, m, -CH2CH2CH2CH2CH2CH3), 0.87 (3H, t, J = 6.8Hz, -CH2CH2CH2CH2CH2CH3)

[0099] 2- & lt; / RTI & gt; methylenenol (2f)

[0100] Using the same method as above, 2f (44-62%) of yellow liquid material was obtained.

[0101] 1(1H, s, -C = CHαHβ), 5.98 (1H, s, -C = CHαHβ), 2.22 (2H, t, M, -CH2 (CH2) 4CH2CH3), 0.87 (3H, t, J = 7.3 Hz, = 6.8 Hz, -CH2 (CH2) 4CH2CH3).

[0102] 2- benzyl acrylidihydrate (2 g)

[0103] Using the same method as above, 2 g (51%) of a yellow liquid material was obtained.

[0104] 1(1H, s, -CH = H), 6.01 (1H, s, , -C? CH? H?), 3.50 (2H, s, Ar-CH2C = CH2).

[0105] & Lt; Example 2 & gt;

[0107] Preparation of (S) -3 - ((2S, 3S) -3-hydroxy-2,4-dimethylpent-4-enoyl) -4- isopropyloxazolidin-

[0108] (R) - (+) 4-isopropyl-3-propynol-2-oxazolidinone 3a (100 mg, 0.541 mmol, 1 eq) dissolved in dichloromethane (1.1 ml) was ice-cooled in an ice water bath (596, 0.596 mmo) and diisopropylethylamine (90.6, 0.650 mmol, 1.2 eq) dissolved in 1 M dichloromethane were slowly added dropwise and stirred at the same temperature for 50 minutes . The stirred reaction mixture was cooled at -78 [deg.] C for 10 minutes, and then methyl acrolein 2a (59.0, 0.704 mmol) dissolved in dichloromethane (0.70 ml) was stirred at -78 [deg.] C for 30 minutes. Then, the mixture was ice-cooled again in an ice water bath and stirred for 1 hour. Then, pH 7.0 phosphate (1.2 ml), methanol (1.1 ml), aqueous 30% wt hydrogen peroxide aqueous solution (0.6 ml) The reaction is stopped in order to stop the reaction. The mixture was further stirred in an ice water bath for 1 hour, and then dichloromethane and water were added to separate the organic

layer. The separated organic layer was washed with an aqueous solution of sodium chloride, dried over anhydrous magnesium sulfate and concentrated. The obtained residue was subjected to silica gel column chromatography (18% n-hexane: ethyl acetate) to separate the material and dry to obtain yellow liquid material 4a (68 mg, 49%).

[0109] 1(1H, m, -OCH? H? CHN), 4.40 (1H, brs, Dd, J = 9.3, 2.9 Hz, -OCH? H? CHN), 3.97 (1H, dq, J = 7.3 , 2.9 Hz, -CH (CH3) CH (OH) -), 3.11 (1H, brs, J = 2.4 Hz, -OH), 2.39-2.32 , 3H, -C (= CH) CH3), 1.18 (3H, d, J = 6.8Hz, -CH (CH3) CH (OH)) 2), 0.89 (3H, d, J = 6.8 Hz, -CH (CH3) 2).

[0110] Preparation of (S) -3- ((2S, 3S) -3-hydroxy-2,5-dimethyl-4-methylenehexanoyl) -4-isopropyloxazolidin-

[0111] Using the same method as above, 4b (43%) of a yellow liquid material was obtained.

[0112] 1(1H, s, -C = CH? H?), 4.48 (1H, m, -OCH? H? CHN), 4.46 Dq, J = 6.8 (1H, d, J = 9.2, 2.9 Hz, -OCH? H? CHN), 4.96 (1H, dd, J = 17.1, 8.3 Hz, M, -CH (CH3) 2), 2.18-2.13 (1H, m, & lt; RTI ID = (3H, d, J = 6.3 Hz, -C (= CH) CH (CH3) 2), 1.19 CH (CH3) 2), 0.93 (3H, d, J = 6.8Hz, -CH (CH3) 2), 0.89 (3H, d, J = 7.3 Hz, -CH (CH3) 2).

[0113] Preparation of (S) -3- ((2S, 3S) -3-hydroxy-2-methyl-4-methyleneoctanoyl) -4-isopropyloxazolidin-

[0114] Using the same method as above, 4c (16%) of a yellow liquid material was obtained.

[0115] 1M, -OCH? H? CHN), 4.41 (1H, brs, 1H), 4.97 (1H, -CH (OH)), 4.28 (1H, dd, J = 17.6,9.2 Hz, -OCH? H? CHN), 4.22 (1H, dd, J = 9.3, 2.9 Hz, , 2.9 Hz, -CH (CH3) CH (OH) -), 3.13 (1H, brs, -OH), 2.38-2.33 (2H, m, -C (= CH) CH2CH2CH2CH3), 1.17 (3H, d, J = 6.8 CH (OH) -), 0.91 (3H, d, J = 6.8Hz, -CH (CH3) 2), 0.87 (3H, d, J = 7.3Hz,), 0.86 (3H, d, J = 7.3Hz, -CH (CH3) 2), 0.85 (3H, t, J = 5.6Hz, -C (= CH)

CH2CH2CH2CH3).

[0116] Preparation of (S) -3- ((2S, 3S) -3-hydroxy-2-methyl-4-methylenonanoyl) -4-isopropyloxazolidin-

[0117] Using the same method as above, 4d (84%) of yellow liquid material was obtained.

[0118] 1(1H, s, -C = CH? H?), 4.50-4.47 (1H, m, -OCH? H? CHN), 4.42 Dq, J = 9.3, 2.9 Hz, -OCH? H? CHN), 3.95 (1H, d, J = (1H, m, -CH (CH3) 2), 2.04-1.90 (2H, m) M, -C (= CH) CH2CH2CH2CH2CH3), 1.18 (3H, d, CH3CH2CH2CH3) J = 7.3 Hz, -CH (CH3) CH (OH) -), 0.93 (3H, d, J = 7.1 Hz, -CH (CH3) CH3) 2), 0.88 (3H, t, J = 5.4 Hz, -C (= CH) CH2CH2CH2CH2CH3).

[0119] Preparation of (S) -3- ((2S, 3S) -3-hydroxy-2-methyl-4-methylenedecanoyl) -4-isopropyloxazolidin-

[0120] 4e (87%) of a yellow liquid material was obtained using the same method as above.

[0121] 1(1H, s, -C = CH? H?), 4.50-4.47 (1H, m, -OCH? H? CHN), 4.42 dd, J = 9.3, 2.9 Hz, -OCH? H? CHN), 3.95 (1H, dq, J = (1H, m, -CH (CH3) 2), 2.04-1.90 (2H, m, -C (= CH) CH2CH2CH2CH2CH2CH3), 1.18 (3H, d, D, J = 7.1 Hz, -CH (CH3) 2), 0.98 (3H, d, J = (CH3) 2), 0.88 (3H, t, J = 5.4 Hz, -C (= CH) CH2CH2CH2CH2CH2CH3).

[0122] Preparation of (S) -3 - ((2S, 3S) -3-hydroxy-2-methyl-4-methylene undecanoyl) -4- isopropyloxazolidin-

[0123] Using the same method as above, 4f (41%) of a yellow liquid material was obtained.

[0124] 1(1H, m, -OCH? H? CHN), 4.42 (1H, s, -C = CH? H?), dd, J = 9.1, 3.1 Hz, -

OCH? H? CHN), 3.95 (1H, dq, J = M, -CH (CH3) 2), 1.95 (2H, m, & lt; RTI ID = M, -C (= CH) CH2 (CH2) 5CH3), 1.18 (3H, d, J = 7.1 Hz, -CH (CH3) CH (OH) -), 0.93 (3H, d, J = 7.0 Hz, -CH (CH3) 2), 0.89 (3H, d, J = 6.72 Hz, , J = 5.8 Hz, -C (= CH) CH2 (CH2) 5CH3).

[0125] Preparation of (S) -3 - ((2S, 3S) -4-benzyl-3-hydroxy-2-methylpent-4-enoyl) -4- isopropyloxazolidin-

[0126] Using the same method as above, 4 g (72%) of a yellow liquid material were obtained.

[0127] 1(1H, s, -C = CHαHβ), 4.42-4.41 (1H, m, (1H, d, J = 9.0, 8. 3 Hz, -OCH? H? CHN), 4.19 (1H, dd, J = 9.0, 3.2 Hz, -OCH? H? CHN), 4.01 (3H, dq, J = 7.1, 3.0 Hz, -CH (CH3) M, -CH (CH3) 2), 3.10 (1H, brs, -OH), 2.35-2.29 , 1.22 (3H, d, J = 7.1Hz, -CH (CH3) CH (OH) -), 0.91 = 6.8 Hz, -CH (CH3) 2).

[0128] & Lt; Example 3 & gt;

[0130] (R) - (+) 4-isopropyl-3-propynol-2-oxazolidinone 3a (100 mg, 0.541 mmol, 1 eq) dissolved in dichloromethane (1.5 ml) was ice-cooled in an ice water bath (596, 0.596 mmo) and diisopropylethylamine (90.6, 0.650 mmol, 1.2 eq) dissolved in 1 M dichloromethane were slowly added dropwise and the mixture was stirred at the same temperature for 50 minutes Lt; / RTI & gt; The stirred reaction mixture was cooled at -78 ° C for 10 minutes, then 1-cyclohexenecarboxylic aldehyde 2h (92.25, 0.81 mmol) dissolved in dichloromethane (0.70 ml) was added dropwise at -78 ° C for 30 minutes Lt; / RTI & gt; Then, the mixture was ice-cooled again in an ice water bath and stirred for 1 hour. Then, pH 7.0 phosphate (1.2 ml), methanol (1.1 ml), aqueous 30% wt hydrogen peroxide aqueous solution (0.6 ml) The reaction is stopped in order to stop the reaction. The mixture was further stirred in an ice water bath for 1 hour, and then dichloromethane and water were added to separate the organic layer. The separated organic layer was washed with an aqueous solution of sodium chloride, dried over anhydrous magnesium sulfate and concentrated. The resulting residue was subjected to silica gel column chromatography (18% n-hexane: ethyl acetate) to isolate the material and dry to obtain yellow liquid material 4h (22.3 mg, 14.8%).

[0131] 1 1 H-NMR (CDCl 3, 500 MHz)? 5.81 (1H, t, J = 3. 4 (1H, d, J = 17.4, 9.0 Hz, -OCH? H? CHN), 4.40 , 2.93 (1H, brs, -), 4.22 (1H, dd, J = 9.3, 2.9 Hz, -OCH? H? CHN), 3.95 (1H, dq, J = 7.3, 2.9 Hz, M, -Cyclo (CH 2)), 1.927 (2H, t, J = 17.60 Hz, -Cyclo (CH 2)), 1.67-1.55 (4H, m, -Cyclo (CH2CH2), 1.18 (3H, d, J = 7.3 Hz, -CH (CH3) 2), 0.88 (3H, d, J = 7.1 Hz, -CH (CH3) 2).

[0132] & Lt; Example 4 & gt;

[0134] 3-Propynol-2-oxazolidinone 3b (100 mg, 0.541 mmol, 1 eq), dissolved in dichloromethane (2.0 ml) (596, 0.596 mmo) and diisopropylethylamine (90.6, 0.650 mmol, 1.2 eq) dissolved in 1 M dichloromethane were slowly added dropwise and the mixture was stirred at the same temperature for 50 minutes Lt; / RTI & gt; The stirred reaction mixture was cooled at -78 ° C for 10 minutes, and 2-methyleneheptal 2d (68.14, 1.07 mmol) dissolved in dichloromethane (0.70 ml) was stirred at 78 ° C for 30 minutes. Then, the mixture was ice-cooled again in an ice water bath and stirred for 1 hour. Then, pH 7.0 phosphate (0.1 ml), methanol (0.2 ml), aqueous 30% wt hydrogen peroxide aqueous solution (0.2 ml) The reaction is stopped in order to stop the reaction. The mixture was further stirred in an ice water bath for 1 hour, and then dichloromethane and water were added to separate the organic layer. The separated organic layer was washed with an aqueous solution of sodium chloride, dried over anhydrous magnesium sulfate and concentrated. The obtained residue was subjected to silica gel column chromatography (15% n-hexane: ethyl acetate) to isolate the material and dry to obtain yellow liquid material 5 (146.3 mg, 87.2%).

[0135] 1(1H, s, -C = CH? H?), 4.50-4.47 (1H, m, -OCH? H? CHN), 4.42 dd, J = 9.3, 2.9 Hz, -OCH? H? CHN), 3.95 (1H, dq, J = (1H, m, -CH (CH3) 2), 2.04-1.90 (2H, m, -C (= CH) CH2CH2CH2CH2CH3), 1.18 (3H, d, D, J = 7.1 Hz, -CH (CH3) 2), 0.98 (3H, d, J = (CH3) 2), 0.88 (3H, t, J = 5.4 Hz, -C (= CH) CH2CH2CH2CH2CH3).

[0136] & Lt; Example 5 & gt;

[0138] 4-benzyl-3-propionyloxazolidin-2-one 6 (100 mg, 0.43 mmol, 1 eq) dissolved in dichloromethane (1.5 ml) was ice-cooled in an ice water bath, Dibutylboron trifluoromethane sulphonate (515, 0.51 mmol) and diisopropylethylamine (104, 0.60 mmol, 1.4 eq) dissolved in dichloromethane were slowly added dropwise and stirred at the same temperature for 50 minutes. The stirred reaction mixture was cooled at -78

° C for 10 minutes, and 2-methyleneheptal 2d (68.14, 1.07 mmol) dissolved in dichloromethane (0.70 ml) was stirred at 78 ° C for 30 minutes. Then, the mixture was ice-cooled again in an ice water bath and stirred for 1 hour. Then, pH 7.0 phosphate (0.1 ml), methanol (0.2 ml), aqueous 30% wt hydrogen peroxide aqueous solution (0.2 ml) The reaction is stopped in order to stop the reaction. The mixture was further stirred in an ice water bath for 1 hour, and then dichloromethane and water were added to separate the organic layer. The separated organic layer was washed with an aqueous solution of sodium chloride, dried over anhydrous magnesium sulfate and concentrated. The obtained residue was separated and dried by silica gel column chromatography (18% n-hexane: ethyl acetate) to obtain yellow liquid material 7a (105 mg, 68%).

[0139] 1(2H, m, Ar-H), 5.19 & lt; RTI ID = 0.0 & gt; (1H, s, -C = CH? H?), 5.00 (1H, s, -C = CH? H?), 4.74-4.69 (1H, m, -OCH? H? CHN) (1H, m, PhCH2-), 3.96 (1H, dd, J = 10.2, 3.4 Hz, -OCH.alpha.H.beta.CHN), 3.27 (1H, dd, J = 13.2, 3.4 Hz,), 2.81 (1H, d, J = 13.2, 9.2 Hz, -CH (CH3) m, -C (= CH) CH2 (CH2) 2CH2CH3), 1.32 (4H, m, CH (CH2) 2CH2CH3), 0.90 (3H, t, J = 6.8Hz,

[0140] The residue obtained in Example 5 was subjected to silica gel column chromatography (18% n-hexane: ethyl acetate) to obtain 7a, and then (7%) (21 mg, 14%) in (12% n-hexane: ethyl acetate).

[0141] 1(2H, m, Ar-H), 7.30-7.27 (1H, m, Ar-H), 7.22-7.21 , 5.19 (1H, s, -C = CH? H?), 5.00 (1H, s, -C? CH? H?), 4.74-4.69 (2H, m, PhCH2-), 3.95 (1H, dd, J = 10.2, 3.4 Hz, -OCH.alpha.H.beta.CHN), 3.27 (1H, dd, J = 13.7, 3.6 Hz, -OCH.alpha.H.beta.CHN) (CH2) 2CH2CH3), 2.00 (2H, m, -C (= CH) CH2 (CH2) 2CH2CH3), 2.81 (1H, dd, J = 13.2, 9.8 Hz, 1.48 (2H, m, -C (= CH) CH2 (CH2) 2CH2CH3), 1.32 (4H, m, CH (CH3) CH (OH) -), 0.90 (3H, t, J = 6.8 Hz, -C (= CH) CH2 (CH2) 2CH2CH3).

[0142] & Lt; Example 6 & gt;

[0144] 3-propynol-2-oxazolidinone 3a (100 mg, 0.541 mmol, 1 eq), dissolved in dichloromethane (1.5 ml) (596, 0.596 mmol) and diisopropylethylamine (90.6, 0.650 mmol, 1.2 eq) dissolved in 1 M dichloromethane were slowly added dropwise and the mixture was stirred at the same temperature for 50 minutes Lt; / RTI & gt; The stirred reaction mixture was cooled at -78 [deg.] C for 10 min, then hectoral 8 (92.25, 0.81

mmol) dissolved in dichloromethane (0.70 ml) was stirred at -78 [deg.] C for 30 min. Then, the mixture was ice-cooled again in an ice water bath and stirred for 1 hour. Then, pH 7.0 phosphate (1.2 ml), methanol (1.1 ml), aqueous 30% wt hydrogen peroxide aqueous solution (0.6 ml) The reaction is stopped in order to stop the reaction. The mixture was further stirred in an ice water bath for 1 hour, and then dichloromethane and water were added to separate the organic layer. The separated organic layer was washed with an aqueous solution of sodium chloride, dried over anhydrous magnesium sulfate and concentrated. The obtained residue was subjected to silica gel column chromatography (18% n-hexane: ethyl acetate) to isolate the substance and dry to obtain yellow liquid substance 9 (22.3 mg, 14.8%).

[0145] 1(1H, m, -OCH? H? CHN), 4.39 (1H, brs, Dd, J = 7.8, 2.9 Hz, -OCH? H? CHN), 3.94 (1H, dq, J = 6.8 M, -CH (CH3) CH (OH) -), 2.38-2.33 (1H, m, -CH (CH3) 1.17 (3H, d, J = 7.3 Hz, -CH (CH3) CH (OH) D, J = 7.3 Hz, -CH (CH3) 2), 0.87 (3H, t, J = 5.8 Hz, -CH? H? CH 2 (CH 2) 3 CH 3).

[0146] & Lt; Example 7 & gt;

[0148] (2S, 3S) -3-hydroxy-2-methyl-4-methylenenanoic acid (10d)

[0149] (S) -3 - ((2S, 3S) -3-hydroxy-2-methyl-4-methylenonanoyl) -4-isopropyloxazolidin- mg, 3.05 mmol) was dissolved in a mixture of tetrahydrofuran: water (27 ml: 27 ml) and lithium hydroxide (256 mg, 6.10 mmol) was added at room temperature. The reaction was completed by stirring at room temperature for 12 hours. Tetrahydrofuran was removed under reduced pressure and neutralized to pH 7. Ethyl acetate was added to the reaction mixture to separate the organic layer. The separated organic layer was washed with an aqueous solution of sodium chloride, dried over anhydrous magnesium sulfate and concentrated. The resulting liquid material 10d (647 mg, 106.1%) was added directly to the next reaction.

[0150] 1(1H, d, J = 3.4 Hz, -CH (CH3) 2 (CH3) (2H, m, -CH2CH2CH2CH2CH3), 1.48 (2H, m, -CH2CH2CH2CH2CH3), 1.31 (4H, m, -CH2CH2CH2CH2CH3), 1.14 (3H, d, J = 37.3 Hz, -CH (CH3) CH-OH), 0.90 (3H, t, J = 6.8 Hz, -CH2CH2CH2CH2CH3).

[0151] & Lt; Example 8 & gt;

[0153] 2- (indolin-3-yl) ethanol (11)

[0154] 2- (3-indol) -ethanol (1.2 g, 7.06 mmol) dissolved in acetic acid (41.5 ml) was ice-cooled in an ice bath and sodium thianoborohydride (2.2 g, 35.34 mmol) . The ice bath was removed and the mixture was stirred at room temperature for 3 hours. After confirming that the reaction was completed, the reaction was stopped by adding a saturated sodium bicarbonate aqueous solution. The reaction mixture was neutralized, and then ethyl acetate was added thereto to separate an organic layer. The separated organic layer was washed with an aqueous solution of sodium chloride, dried over anhydrous magnesium sulfate and concentrated. The obtained residue was subjected to silica gel column chromatography (n-hexane: ethyl acetate = 10: 1) and the material was separated and dried to obtain a yellow liquid substance 11 (432 mg, 37.4%).

[0155] 1(1H, dd, J = 7.6 Hz), 7.06 (1H, dd, J = 7.9, 7.3 Hz), 6.76 , 6.68 (1H, d, J = 7.6Hz), 3.74-3.55 (3H, m), 3.44 2.09 (1 H, m), 1.79 (1 H, m)

[0156] 3- (2- (tert-butyldimethylsilyloxy) ethyl) indoline (12)

[0157] 2 - (indolin-3-yl) ethanol 11 (365 mg, 2.23 mmol) was dissolved in dichloromethane and then imidazole (182.6 mg, 2.68 mmol) and tert- butyl dimethylsilyl chloride 404.2 mg, 2.68 mmol) were added successively. After stirring at room temperature for 3 hours, it was confirmed that the reaction was completed. The reaction mixture was poured into water and dichloromethane to quench the reaction, and the organic layer was separated. The separated organic layer was washed with an aqueous solution of sodium chloride, dried over anhydrous magnesium sulfate and concentrated. The obtained residue was subjected to silica gel column chromatography (n-hexane: ethyl acetate = 20: 1) and the material was separated and dried to obtain yellow liquid material 12 (570.5 mg, 92.8%).

[0158] 1(1H, dd, J = 7.6 Hz), 7.06 (1H, dd, J = 7.9, 7.3 Hz), 6.76 , 6.68 (1H, d, J = 7.6 Hz), 3.74-3.55 (3H, m), 3.44 1.79 (1H, m), 0.92 (9H, s, -Si- (CH3) 2- (CH3) 3), 0.09 (6H, s, -Si- (CH3) 2- (CH3) 3).

[0159] & Lt; Example 9 & gt;

[0161] (2S, 3S) -1- (3- (2- (tert-butyldimethylsilyloxy) ethyl) indolin-1-yl) -3-hydroxy-2-methyl- (13a)

[0162] 3 (405 mg, 1.45 mmol) and (2S, 3S) -3-hydroxy-2-methyl-4-methylrenonexecarboxylic acid (438 mg , 2.18 mmol) and O- (7-azabenzotriazol-1-yl) -N, N, N ', N'- tetramethyl- uranium (832.4 mg, 2.18 mmol) were dissolved in 20.0 ml of dimethylformamide , N, N-diisopropylethylamine (0.17 ml, 1.0 mmol) was added. The organic layer was dried over anhydrous magnesium sulfate, concentrated and then purified by silica gel column chromatography (n-hexane: ethyl acetate = 10: 1) to obtain the desired compound 13a (404 mg, 60.2%) as an oil.

[0163] 1(2H, m, Ar-H), 7.09 (1H, t, J = 7.3 Hz, Ar (1H, brs, HC-OH), 5.27 (1H, t, J = 6.35 Hz, (2H, m, -N-CH2), 3.56-3.53 (1H, m, -CO-OTBS), 3.92 (1H, q, J = 5.86,4.4 Hz, M, -CH2-CH2-OTBS), 2.91 (1H, m, -C? CH = CH? H? -CH2), 1.82 (1H, m, -CH2CH2CH2CH3), 1.21 (3H, d, J = 6.84 Hz, C = O -CH (CH3) 3), 0.09 (6H, s, -Si-(CH3) 3), 1.19 (3H, t, J = 7.33 Hz, CH2CH3) CH3) 2- (CH3) 3).

[0164] (2Preparation of (S) -3-hydroxy-1- (3- (2-hydroxyethyl) indolin-1-yl)

[0165] (2 3-hydroxy-2-methyl-4-methylenenan-1-one 13a ((S) 54.5 mg, 0.12 mmol) was dissolved in tetrahydrofuran (5 ml), and tetrabutylammonium fluoride (0.15 ml) dissolved in 1.0 M of tetrahydrofuran was added in an ice water bath. After stirring at room temperature for 2 hours, it was confirmed that the reaction was completed. Methanol was added to the reaction mixture to stop the reaction, and the obtained residue was immediately subjected to silica gel column chromatography (n-hexane: ethyl acetate = 1: 1) to separate the material and dry to obtain the desired compound 13b (39 mg, 95% Oil.

[0166] 1(2H, m, Ar-H), 7.08 (1H, t, J = 6.8 Hz, Ar (1H, brs, HC-OH), 4.49 (2H, s, HC-OH), 5.07 (2H, m, -N-CH2), 3.83 (1H, q, J = 5.86, 4.4 Hz, (1H, m, -CH2-CH2-OH), 3.79 (1H, brs, CH2CH2OH), 3.63-3.59 M, -CH2-CH3), 1.34-1.32 (4H, m, -CH2CH2CH2CH2), 1.86 m, -CH2CH2CH2CH3), 1.21 (3H, d, J = 7.3 Hz, C-O-CH (CH3), 0.91 (3H, t, J = 6.84 Hz, CH2CH3).

[0167] (2Ethyl) indolin-1-yl) -2-methyl-4-methylene-1-oxonan-3-yl acetate Manufacturing (14a)

[0168] (2S, 3S) -1- (3- (2- (tert-butyldimethylsilyloxy) ethyl) indolin-1-yl) -3-hydroxy- 1-one 13a (357 mg, 0.77 mmol) was added pyridine (0.34 ml, 3.88 mmol), and then the acetonic hydrosol was slowly added dropwise. The mixture was stirred at room temperature for 12 hours. A small amount of starting material remained, and 4-dimethylaminopyridine (1 eq) was added thereto, followed by stirring at room temperature for 2 hours. After confirming that the reaction was completed, pyridine was removed by a pressure reducer, ethyl acetate and water were added to separate the organic layer. The separated organic layer was washed with an aqueous solution of sodium chloride, dried over anhydrous magnesium sulfate and concentrated. The obtained residue was subjected to silica gel column chromatography (n-hexane: ethyl acetate = 20: 1), and the material was separated and dried to obtain the desired compound 14a (570.5 mg, 92.8%) as an oil.

[0169] 1(2H, m, Ar-H), 6.95 (1H, t, J = 7.3 Hz, Ar (1H, d, J = 8.3 Hz, -CHOCH3), 5.04 (1H, s, -CH2OH-OTBS), 3.44 (1H, m, -CO-NH2), 3.69 CH (CH3), 2.94 (1H, m, Ar-CH), 2.01 M, -CH2-CH3), 0.86 (4H, m, -CH2CH2CH2CH3), 0.85 (9H, s, (3H, t, J = 7.33 Hz, CH2CH3), 0.01 (6H, dd, J = , s, -Si- (CH3) 2- (CH3) 3).

[0170] (2Preparation of (14b) S, 3S) -1- (3- (2-hydroxyethyl) indolin-

[0171] (2 Yl) -2-methyl-4-methylene-1-oxononan-3-ylacetate 14a (2-tert-butyldimethylsilyloxy) ethyl) indolin- (64.4 mg, 0.12 mmol) was dissolved in methanol (3 ml), and a small amount of pyridine p-toluene-reductone was added thereto, followed by stirring at room temperature for 2 hours. After confirming that the reaction had been completed, the methanol was blown off with a decompressor and then the material was separated by silica gel column chromatography (n-hexane: ethyl acetate = 1: 1) and dried to obtain the desired compound 14b (21.3 mg, 42.8% State.

[0172] 1(2H, m, Ar-H), 7.05 (1H, t, J = 7.3 Hz, Ar (1H, d, J = 8.3 Hz, -CHOCH3), 5.12 (1H, s, (2H, t, J = 9.2 Hz, -CH2CH2-OH), 3.94 (2H, m, -CH2CH2CH2CH2CH3), 1.45 (4H, CH), 3.04 (1H, t, J = 6.8 Hz, , m, -CH2CH2CH2CH2CH3), 1.26 (3H, m, CO

(CH3) CH), 0.86 (3H, t, J = 6.3Hz, -CH2CH2CH2CH2CH3).

[0173] (2Yl) -2-methyl-4-methylene-1-oxonan-3-yl Acetate (15a)

[0174] (2 Yl) -2-methyl-4-methylene-1-oxononan-3-ylacetate 14a (2-tert-butyldimethylsilyloxy) ethyl) indolin- (344 mg, 0.68 mmol) was dissolved in benzene (7 ml) followed by the addition of 2,3-dichloro-5,6-dithiano-1,4-benzocycnone (778.2 mg, 3.42 mmol) Gt; C & lt; / RTI & gt; for 12 hours. After confirming that the reaction was completed, ethyl acetate and water were added to separate the organic layer. The separated organic layer was washed with an aqueous solution of sodium chloride, dried over anhydrous magnesium sulfate and concentrated. The resulting residue was purified by silica gel column chromatography (n-hexane: ethyl acetate = 7: 1), and the material was isolated and dried to obtain the desired compound 15a (85 mg, 24.8%) as an oil.

[0175] 1(2H, m, Ar-H), 7.72-7.70 (1H, m, Ar-H) (1H, d, J = 7.3 Hz, -CHOCH3), 5.12 (1H, s, (2H, m, -CH2CH2-OTBS), 2.11 (3H, s, CHOCOCH3), 3.93 (2H, m, -CH2CH2CH2CH2CH3), 1.38 (3H, d, J = 7.3 Hz, -CO-CH (CH3), 1.32-1.23 4H, m, -CH2CH2CH2CH2CH3), 0.90 (9H, s, -Si- (CH3) 2- (CH3) 3), 0.84 (3H, t, J = 7.3Hz, -CH2CH2CH2CH2CH3) = 4.89 Hz, -Si- (CH3) 2- (CH3) 3).

[0176] (2Methyl-4-methylene-1-oxononan-3-ylacetate (15b) & lt; EMI ID =

[0177] (2 Yl) -2-methyl-4-methylene-1-oxonan-3-yl Acetate 15a (88.3 mg, 0.17 mmol) was dissolved in tetrahydrofuran (5 ml) and tetrabutylammonium fluoride (1.76 ml) dissolved in 1.0 M of tetrahydrofuran was added thereto in an ice water bath . After stirring at room temperature for 2 hours, it was confirmed that the reaction was completed. An aqueous ammonium chloride solution was added to the reaction mixture to stop the reaction, and ethyl acetate was added to separate the organic layer. The separated organic layer was washed with an aqueous solution of sodium chloride, dried over anhydrous magnesium sulfate and concentrated. The resulting residue was purified by silica gel column chromatography (n-hexane: ethyl acetate = 1: 1) and the material was isolated and dried to obtain the desired compound 15b (21.3 mg, 31.3%) as an oil.

[0178] 1(1H, d, J = 7.8 Hz, Ar-H), 7.55 (1H, d, J = 6.84 Hz, Ar-H), 7.38 = 7.3 Hz,

Ar-H), 7.37 (1H, s, Ar-H), 7.30 (1H, t, J = 7.3 Hz, Ar- (2H, m, 3H), 5.11 (1H, s, -C = CH? H?), 4.94 (2H, m, CH 2 CH 2 OH), 2.09 (3H, s, CH 2 CH 2), 3.69 CHOCH3), 2.05 (2H, s, CH2CH2CH2CH2CH3), 1.42-1.52 (6H, m, CH2CH2CH2CH2CH3), 0.85 (3H, m, -CH2CH3).

[0179] <Example 10>

[0181] (2Ethyl) -1H-indol-1-yl) -3-hydroxy-2-methyl-4-methylenenan-1-one (16a)

[0182] (2 3-hydroxy-2-methyl-4-methylenenan-1-one 13a ((S) 156 mg, 0.31 mmol) was dissolved in benzene (30 ml), followed by the addition of 2,3-dichloro-5,6-dithianano-1,4-benzocycnone (352.8 mg, 1.55 mmol) C & lt; / RTI & gt; for 12 hours. After confirming that the reaction was completed, ethyl acetate and water were added to separate the organic layer. The separated organic layer was washed with an aqueous solution of sodium chloride, dried over anhydrous magnesium sulfate and concentrated. The obtained residue was subjected to silica gel column chromatography (n-hexane: ethyl acetate = 20: 1) to separate the material and dry to obtain the desired compound 16a (102 mg, 73.9%) as an oil.

[0183] 1(1H, d, J = 7.8 Hz, Ar-H), 7.38 (1H, t, J = = 8.31 Hz, Ar-H), 7.33 (1H, s, Ar-H), 7.3 (1H, t, J = 6.35 Hz, Ar-H), 5.28 M, -HC-OH), 3.31 (2H, t, J = 6.35 Hz, -CH2-OTBS) (1H, m, -CO-CH (CH3)), 2.94 (1H, t, J = 6.84 Hz, -CH.alpha.H.beta.-OTBS), 2.93 (H, m, -CH2CH2CH2CH2CH3), 1.35 (3H, s, -CO (CH2CH2CH2CH2) -CH (CH3) 3), 0.03 (6H, s, -Si- (CH3) 2), 1.34 (4H, m, -CH2CH2CH2CH2CH3), 0.89 - (CH3) 3).

[0184] (2Preparation of (S), 3S) -3-hydroxy-1- (3- (2-hydroxyethyl) -1H-indol-

[0185] (2 Ethyl) -1H-indol-1-yl) -3-hydroxy-2-methyl-4-methylenenan-1-one 16a (9.9 mg, 0.02 mmol) was dissolved in methanol (2 ml), and a small amount of pyridine p-toluene-reductone was added thereto, followed by stirring at room temperature for 2 hours. After confirming that the reaction was completed, the methanol was blown off with a pressure reducing device and then the material was separated by

silica gel column chromatography (n-hexane: ethyl acetate = 1: 1) and dried to obtain the desired compound 16b (6.9 mg, 94.5% State.

[0186] 1(1H, d, J = 7.8 Hz, Ar-H), 7.41-7.31 (3H, m, (1H, s, C = OCH (CH3) CH-OH), 3.98 (2H, (t, J = 5.8 Hz, -CH2-OTBS), 3.51 (1H, brs, CH-OH), 3.34-3.29 , -CH2CH2-OTBS), 2.12-2.06 (1H, m, -C = CH.alpha.H.beta.), 2.00-1.94 M, -CH2CH2CH2CH2CH3), 0.90 (3H, t, J = 5.8 Hz, -CH2CH2CH2CH3), 1.36 , CH2CH3).

[0187] (2Preparation of (R), 3S) -1- (3- (2- (tert-butyldimethylsilyloxy) ethyl) indolin-

[0188] (2 3-hydroxy-2-methyl-4-methylenenan-1-one 13a ((S) 250 mg, 0.54 mmol) was dissolved in tetrahydrofuran (15 ml), and then borane-dimethylsulfide (0.5 ml) dissolved in tetrahydrofuran at 2M was added thereto while ice-cooled. After confirming that the reaction is completed by stirring in an ice water bath for 2 hours, add methanol (1 ml) and stir for 3 hours. The reaction mixture was blown off using a pressure reducer, followed by silica gel column chromatography (n-hexane: ethyl acetate = 15: 1) and the material was isolated and dried to obtain 178 mg (73.4%) of the desired compound 17a as an oil.

[0189] 1T, J = 7.8 Hz, Ar-H), 6.66 (1H, t, J = 7.3 Hz, Ar- (1H, d, J = 8.3 Hz, -CH (CH3) CH- OH), 3.75 (2H, d, J = 5.86 Hz, N-CH2), 3.64-3.54 CH (CH3), 2.84 (1H, m, CH2CH (CH3), 3.08-2.98 (1H, m, (2H, m, -CH2CH2CH2CH2CH3), 1.47 (2H, m, CH3), 2.05 (2H, q, J = 7.8Hz, CH2CH2- (CH3) 2-CH3), 0.92 (9H, s, -Si- (CH3) 2-(CH3) 3), 0.89 (3H, t, J = 6.8Hz, -CH2CH2CH2CH2CH3), 0.09 (6H, s, -Si- (CH3) 2-(CH3) 3).

[0190] (2Preparation of (R, 3S) -1- (3- (2-hydroxyethyl) indolin-1-yl)

[0191] (2 Yl) -2-methyl-4-methylenone-3-ol 17a (31 mg, 0.06 mmol,) Was dissolved in methanol (1.3 ml), and a small amount of pyridine p-toluene-reductone was added thereto, followed by stirring at room temperature for 3 hours. After confirming that the reaction was completed, the methanol was blown off with a decompressor, followed by silica gel column chromatography (n-hexane: ethyl acetate = 1: 1), followed by separation of the material and drying to obtain the desired

compound 17b (18.4 mg, 79.8% Oil.

[0192] 1(1H, m, Ar-H), 6.70 (1H, t, J = 7.3 Hz, Ar- D, J = 7.3 Hz, CH (CH3) CH-OH), 3.77 (1H, s, - 3.71 (2H, m, N-CH2CH (CH3)), 3.58 (1H, t, J = 8.3 Hz, N- (2H, m, N-CH2), 2.92 (1H, m, N-CH2CH (CH3), 2.05 m, -CH2CH2CH2CH3), 0.91 (3H, s, -CH2CH (CH3), m, CH2CH2-OH), 1.83 (2H, m, = C-CH2CH2CH2CH3), 1.47), 0.89 (3H, t, J = 7.3Hz, -CH2CH2CH2CH2CH3).

[0193] (2Preparation of (R), 3S) -1- (3- (2- (tert-butyldimethylsilyloxy) ethyl) -lH-indol-

[0194] (2 Yl) -2-methyl-4-methylenenan-3-ol 17a (87.8 mg, 0.19 mmol,) Was dissolved in benzene (18 ml), 2,3-dichloro-5,6-dithianano-1,4-benzocycnone (89.43 mg, 0.39 mmol) was added and the mixture was stirred at 60 ° C for 12 hours Lt; / RTI & gt; After confirming that the reaction was completed, ethyl acetate and water were added to separate the organic layer. The separated organic layer was washed with an aqueous solution of sodium chloride, dried over anhydrous magnesium sulfate and concentrated. The obtained residue was subjected to silica gel column chromatography (n-hexane: ethyl acetate = 20: 1) to separate the material and dry to obtain the desired compound 18a (32 mg, 36.6%) as an oil.

[0195] 17.38 (1H, d, J = 7.3 Hz, Ar-H), 7.18 (1H, t, (1H, d, J = 8.3 Hz, Ar-H), 7.08 (1H, t, J = 7.8 Hz, Ar-H), 6.97 (1H, s, -C = CH? H?), 4.21 (1H, q, J = 8.3 Hz, N-CHαHβCH (CH3)), 3.99 (1H, q, J = 8.3 Hz, N-CHαHβCH (2H, t, J = 7.3 Hz, CH2CH2-OTBS), 3.83 (1H, d, J = 6.3 Hz, CH M, -CH2CH2CH2CH3), 0.90 (1H, m, -CH2CH2CH2CH3), 2.27 (1H, (3H, t, J = 7.3Hz, -CH2CH2CH2CH2CH3), 0.04 (6H, CH3) s, -Si- (CH3) 2- (CH3) 3)).

[0196] (2Preparation of (R, 3S) -1- (3- (2-hydroxyethyl) -1H-indol-

[0197] (2 R, 3S) -1- (3- (2- (tert-butyldimethylsilyloxy) ethyl) -lH-indol- 0.08 mmol) was dissolved in tetrahydrofuran (4 ml), and then terephthaloylammonium fluoride dissolved in 1.0 M of tetrahydrofuran was added in an ice water bath. After stirring at room temperature for 2 hours, it was confirmed that the reaction was completed. An aqueous ammonium chloride solution was added to the reaction mixture

to stop the reaction, and ethyl acetate was added to separate the organic layer. The separated organic layer was washed with an aqueous solution of sodium chloride, dried over anhydrous magnesium sulfate and concentrated. The resulting residue was purified by silica gel column chromatography (n-hexane: ethyl acetate = 1: 1) and the material was separated and dried to obtain the desired compound 18b (12.02 mg, 36%) as an oil.

[0198] 1(1H, d, J = 7.8 Hz, Ar-H), 7.38 (1H, d, J = 8.3 Hz, Ar- J = 8.3 Hz, Ar-H), 7.11 (1H, t, J = 7.8 Hz, Ar-H), 7.04 (1H, s, -C = CHαHβ), 4.23 (1H, q, J = 8.3 Hz, N-CHαHβCH (CH3)), 4.02 (1H, q, J = 7.3Hz, N-CHαHβCH (2H, t, J = 6.3Hz, CH2CH2-OH), 2.31-2.26 (1H, (1H, m, -CH2CH (CH3)), 1.90 (1H, m, C-CHαHβC4H11)), 1.30-1.17 (6H, m, -CH2CH2CH2CH2CH3), 0.857 (3H, s, CH2CH (CH3), 0.84 (3H, s, -CH2CH2CH2CH2CH3).

[0199] Experimental Example 1 IL-6 mediated luciferase inhibitory activity

[0200] <1-1> Preparation of transformant

[0201] 96 HepG2 cells (ATCC HB-8065) were dispensed at 5 x 104 cells / well into well plates, and then 10% FBS (v / v), 60.0 mg / l kanamycin sulfate (Gibco. were cultured in DMEM culture medium containing 1 L sodium bicarbonate (NaHCO3; Sigma, USA) at a temperature of 37 ° C and 5% CO 2 until the culture dish was confluent at 80%. Subsequently, the mixture was exchanged with 50 μl of serum-free medium, and a mixture of 0.1 μg pSTAT3-TA-Luc (Clontech, CA) and 0.3 μl lipofectamine reagent (Invitrogen, USA) was added to each well. -TA-Luc was transfected and replaced with freshly prepared 200 [mu] l DMEM culture medium and incubated for an additional 24 hours.

[0202] <1-2> IL-6 and IL-11 reactive STAT3 reporter gene test

[0203] The transfected cells were serum-starvated with 1% BSA / DMEM, treated with 1 ng / ml IL-6 (R & D system, USA) for 3 hours .

[0204] 1: Negative control group (untreated group);

[0205] 2: Positive control (IL-6 10 ng / ml);

[0206] 3: Compounds (0.3, 1, 3, 10, 30, 100 [mu] M); And

[0207] 4: Madindoline (0.3, 1, 3, 10, 30, 100 [mu] M)

[0208] The cells were washed with PBS and mixed with 50 μl lysis buffer (luciferase assay system, promega, USA) for 1 min. Then, 30-100 μl of luciferase assay system (promega, USA) The degree of color development was measured with a luminometer (EG & G BERTHOLD, USA) within 5 minutes.

[0209] The IC50 values of the IL-6-induced luciferase inhibitory activity of each compound were as shown in Fig. 1 and compound 4d showed the IL-11 induced luciferase inhibitory activity as a concentration-dependent manner, and the IC50 value thereof was 2.1 mM.

[0210] <1-3> STAT3 phosphorylation inhibition activity induced by IL-6

[0211] 6 HepG2 cells were plated in a well plate at a density of 5 x 10 & lt; 4 & gt; cells / well and cultured in a culture dish to a full 80%, then exchanged with serum-free medium for another 6 hours and treated for 30 minutes as described below.

[0212] 1: Negative control group (untreated group);

[0213] 2: Positive control (20 ng / ml of IL-6); And

[0214] 3: Compound 4d treated group (1, 3, 0.39, 10, 30 and 100 [mu] M)

[0215] Then, the cells were treated with 20 ng / ml of IL-6 for 10 minutes and then lysed in 40 $\mu\ell$ lysis buffer [pH 8, 20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 1%

Triton X-100, 1 mM Na3VO4, 2 mM EDTA, 1 mM PMSF, 20 mM leupeptin, 20 [mu] g / ml aprotonin; Sigma, USA], followed by centrifugation (13000 g, 15 minutes) to obtain a supernatant in which protein was dissolved. At this time, samples and HepG2 cells not treated with IL-6 were used as a control group. Protein concentration was determined using a DC protein test kit (Bio-Rad, USA) and the protein was loaded onto 10% SDS polyacrylamide gel (SDS-PAGE) and electrophoresed at 30 mA for 2 hours. After electrophoresis, the proteins of the gel were transferred to a PVDF membrane (Westran S, pore size 0.2 mm; Whatman, USA) at 90 V for 90 minutes. The transferred membrane was blocked with Tris-buffer (T-TBS; 50 mM Tri-HCl, pH 7.6, 150 mM NaCl, 0.2% Tween-20, 5% skim milk; Sigma, USA) for 12 hours at 4 ° C And washed 5 times with T-TBS. The membrane was treated with phospho-STAT3 (1: 1000 dilution) polyclonal antibody as primary antibody for 2 hours. After washing 5 times with T-TBS, HRP-conjugated anti-rabbit antibody (1: 5000 dilution) was reacted with secondary antibody for 1 hour. After washing with T-TBS, the film was developed in an dark room using ECL kit (Amersham, USA).

[0216] As a result, as shown in FIG. 3, the compound of Chemical Formula 1 of the present invention showed IL-6-induced STAT3 phosphorylation inhibitory activity.

[0217] <1-4> IL-6-induced JAK2 and gp130 phosphorylation inhibitory activity

[0218] 6 HepG2 cells were plated in a well plate at a density of 5 x 10 & lt; 4 & gt; cells / well and cultured in a culture dish to a full 80%, then exchanged with serum-free medium for another 6 hours and treated for 30 minutes as described below.

[0219] 1: Negative control group (untreated group);

[0220] 2: Positive control (20 ng / ml of IL-6); And

[0221] 3: Compound 4d treated group (1, 3, 0.39, 10, 30 and 100 [mu] M)

[0222] Then, the cells were treated with 20 ng / ml of IL-6 for 10 minutes and then lysed in 40 ㎕ lysis buffer [pH 8, 20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na3VO4, 2 mM EDTA, 1 mM PMSF, 20 mM leupeptin, 20 [mu] g /

ml aprotonin; Sigma, USA], followed by centrifugation (13000 g, 15 minutes) to obtain a supernatant in which protein was dissolved. The anti-JAK2 antibody and anti-gp130 antibody (Cell signaling, INC.) At 4 ° C for 12 hours, and then precipitated with protein A / G plus agarose (Santa Cruz Biotechnology, USA). After centrifugation, the precipitate was washed three times with buffer solution and used as an electrophoresis sample. At this time, IL-6 and non-specimen treated HepG2 cells were used as a control. Protein concentration was determined using a Bio-Rad DC protein assay kit. Proteins were loaded on 8% SDS-polyacrylamide gels (SDS-PAGE) and electrophoresed at 30 mA for 2 h. After electrophoresis, gel proteins were transferred to PVDF membrane (WeatranS, pore size 0.2 mm) at 90 V for 90 minutes. The transferred membrane was blocked with Tris-buffered solution (T-TBS; 50 mM Tri-HCl, pH 7.6, 150 mM NaCl, 0.2% Tween-20, 5% skim milk) for 12 hours at 4 ° C, 5 times. The membranes were treated with polyclonal antibodies of phospho-tyrosine antibodies (1: 1000 dilutions, respectively) as primary antibodies for 2 h. After washing 5 times with T-TBS, HRP-conjugated anti-mouse antibody (1: 5000 dilutions) was reacted with secondary antibody for 1 hour.

After washing with T-TBS, the film was developed with ECL in a dark room. As a result, as shown in FIG. 4, the compound of formula (I) of the present invention showed IL-6 induced JAK2 and gp130 phosphorylation inhibitory activity.

[0223] <1-5> Effect of compound 4d on insulin-induced adipogenesis

[0224] 3 T3L1 cells were replaced with differentiation medium (containing 5 mg / ml insulin, 1 mM dexamethasone, and 0.5 mM IBMX) two days before confluence in a 24 well cell culture plate and changed into differentiation medium every 2 days for 8 days . At this time, cells that did not use a differentiation medium or cells that had been treated with IL-6 or IL-6 alone in the differentiation medium, and cells that were treated with IL-6 and IL-6 in the differentiation medium were compared.

8
The cells were washed with PBS, fixed with PBS solution containing 3.7% formaldehyde, treated with Oil Red O dye and stained for 1 hour. And washed with 25% isopropanol and observed under a microscope. As a result, as shown in FIG. 5, the compound of Chemical Formula 1 of the present invention showed an activity of restoring the IL-6 effect which inhibits insulin-induced adipogenesis.

CLAIMS KR20110047179

1.

Wherein R 1 is hydrogen or substituted or unsubstituted C 1-10 alkyl, and R 2 is hydrogen or a straight-chain or branched alkyl group having 1 to 10 carbon atoms, or a pharmaceutically acceptable salt thereof, , Branched or cyclic alkyl, R3 is hydrogen, or straight chain, branched or cyclic alkyl of C1-10, or substituted or unsubstituted benzyl; R4 is hydrogen, or straight, branched or cyclic alkyl of C1-10, Or unsubstituted benzyl, X is hydrogen, halogen, hydroxy, methoxy, substituted or unsubstituted C1-10 alkyl, Y is hydrogen or oxygen, Z is hydroxy, C1-10 alkoxy, -OCOCH3 Or oxygen; And the above is a single or double bond.

2.

3. A compound according to claim 1, wherein R1 is hydroxyethyl or tert-butyldimethylsilyloxyethyl, R2 is C1-5 straight-chain, branched or cycloalkyl, R3 is hydrogen, X is hydrogen, halogen, hydroxy, methoxy, substituted or unsubstituted alkyl, Y is hydrogen or oxygen, Z is hydroxy, C1-5 alkoxy, -OCOCH3, or oxygen ; And wherein said is a single or double bond, or a pharmaceutically acceptable salt thereof.

3.

The compound of claim 1, wherein the compound is selected from the group consisting of: (1) (2S, 3S) -1- (3- (2- (tert- butyldimethylsilyloxy) ethyl) indolin- (2S, 3S) -1- (3- (2- (tert-butyldimethylsilyloxy) ethyl) indolin-1-yl) (2S, 3S) -1- (3- (2- (tert-butyldimethylsilyloxy) ethyl) -lH- indol- 1 -yl) - methylene-1-oxononan-

(2S, 3S) -1- (3- (2-hydroxyethyl) -1H-indol-1-yl) -2 (2S, 3S) -1- (3- (2-(tert-butyldimethylsilyloxy) ethyl) -1H-indole-1 6) (2S, 3S) -3-hydroxy-1- (3-(2-hydroxyethyl) -1H- (2R, 3S) -1- (3- (2- (tert-butyldimethylsilyloxy) ethyl) indolin-1 Methyl-4-methylenone-3-ol, 8) (2R, 3S) -1- (3- (2- (tert-butyldimethylsilyloxy) ethyl) Yl) -2- 3-ol, 9) (2R, 3S) -1- (3- (2-hydroxyethyl) -1H-indol- 3-ol, 10) (2S, 3S) -3-hydroxy-1- (3- (2- hydroxyethyl) indolin- 11) Synthesis of (2S, 3S) -1- (3- (2-hydroxyethyl) indolin-1-yl) 2R, 3S) -1- (3- (2-hydroxyethyl) indolin-1-yl) -2-methyl-4-methylenenonan-3-ol or a pharmaceutically acceptable salt thereof. Possible salts.

4.

A pharmaceutical composition for the prophylaxis and treatment of inflammatory diseases or cancer, comprising a compound of any one of claims 1 to 3 or a pharmaceutically acceptable salt thereof.

5.

5. The composition of claim 4, wherein the inflammatory disease or cancer is caused by IL-6 or IL-11.

6.

5. The method of claim 4, wherein the inflammatory disease is selected from the group consisting of rheumatoid arthritis, osteoporosis, transglottial hyperplasia, hyperimmunoglobulinemia, anemia, nephritis, cachexia, angiosclerosis, multiple sclerosis, uveitis, chronic thyroiditis, , Systemic lupus erythematosis, Crohn's disease, pancreatitis, psoriasis, burning idiopathic atrophy, diabetes and Alzheimer's.

7.

The method of claim 4, wherein the cancer is selected from the group consisting of pancreatic cancer, breast cancer, prostate cancer, brain tumor, head and neck carcinoma, melanoma, myeloma, melanoma, leukemia, lymphoma, liver cancer, gastric cancer, colon cancer, ovarian cancer, ovarian cancer, Renal cell carcinoma, kidney cell carcinoma, renal pelvic carcinoma, and central nervous system tumor. The present invention also relates to the use of a compound of formula (I) or a pharmaceutically acceptable salt thereof. Wherein the cancer is selected from the group consisting of.

Electronic Acknowledgement Receipt

EFS ID:	33247840
Application Number:	15988463
International Application Number:	
Confirmation Number:	7597
Title of Invention:	TREATMENT OF FIBROSIS
First Named Inventor/Applicant Name:	Stuart Alexander Cook
Customer Number:	23628
Filer:	Amy Jeanette McMahon/Ashley Cerrone
Filer Authorized By:	Amy Jeanette McMahon
Attorney Docket Number:	M0546.70012US01
Receipt Date:	23-JUL-2018
Filing Date:	24-MAY-2018
Time Stamp:	09:42:03
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	M054670012US01-TRN-AM.pdf	25175 de4cd1a217ea5a3401b983bedfe04e6da903f00d	no	1

Warnings:

Information:					
2	Transmittal Letter	M054670012US01-IDSBODY-AM.pdf	26859 2d6479cea37324556add9a730a175b2578c2208b	no	3
Warnings:					
Information:					
3	Information Disclosure Statement (IDS) Form (SB08)	M054670012US01-IDS1449-AM.pdf	21410 f684dc99c46b06728d61e21c000e191ec0d1bc7d	no	1
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
4	Non Patent Literature	Metz_et_al_J-biol_chem_2007_v282_p1238.pdf	673448 09406934793db3b2345f5264ca6d7df277584fa9	no	12
Warnings:					
Information:					
5	Foreign Reference	KR20110047179.pdf	1340881 7a89a02788be34b0c6017dd63033f759570258f4	no	61
Warnings:					
Information:					
			Total Files Size (in bytes):	2087773	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM *(to be used for all correspondence after initial filing)*			
		Application Number	15/988,463-Conf. #7597
		Filing Date	May 24, 2018
		First Named Inventor	Stuart Alexander Cook
		Art Unit	1646
		Examiner Name	Prema Maria Mertz
Total Number of Pages in This Submission		Attorney Docket Number	M0546.70012US01

ENCLOSURES (***Check all that apply***)

☐ Fee Transmittal Form	☐ Drawing(s)	☐ After Allowance Communication to TC
☐ Fee Attached	☐ Licensing-related Papers	☐ Appeal Communication to Board of Appeals and Interferences
☐ Amendment/Reply	☐ Petition	☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
☐ After Final	☐ Petition to Convert to a Provisional Application	☐ Proprietary Information
☐ Affidavits/declaration(s)	☐ Power of Attorney, Revocation Change of Correspondence Address	☐ Status Letter
☐ Extension of Time Request	☐ Terminal Disclaimer	☒ Other Enclosure(s) (please Identify below):
☐ Express Abandonment Request	☐ Request for Refund	PTO Form-1449 Copies of cited references
☒ Information Disclosure Statement	☐ CD, Number of CD(s) _____	
☐ Certified Copy of Priority Document(s)	☐ Landscape Table on CD	
☐ Reply to Missing Parts/ Incomplete Application	Remarks	
☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53		

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Amy J. McMahon/		
Printed name	Amy J. McMahon, PhD		
Date	July 23, 2018	Reg. No.	73,073

Certificate of Electronic Filing under 37 CFR §1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: July 23, 2018 Signature: /Ashley A. Cerrone/ (Ashley A. Cerrone)

6486068.1

DOCKET NO.: M0546.70012US01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Stuart Alexander Cook
Application No.:	15/988,463
Confirmation No.:	7597
Filed:	May 24, 2018
For:	TREATMENT OF FIBROSIS
Examiner:	Prema Maria Mertz
Art Unit:	1646

Certificate of Electronic Filing under 37 CFR §1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: July 23, 2018 Signature: /Ashley A. Cerrone/ (Ashley A. Cerrone)

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT FILED PURSUANT TO THE DUTY OF DISCLOSURE UNDER 37 C.F.R. §§ 1.56, 1.97 AND 1.98

Sir:

Pursuant to the duty of disclosure under 37 C.F.R. §§ 1.56, 1.97 and 1.98, the undersigned requests consideration of this Information Disclosure Statement.

PART I: Compliance with 37 C.F.R. § 1.97

This Information Disclosure Statement has been filed within three months of the filing date of a national application other than a continued prosecution application under 37 C.F.R. § 1.53(d).

No fee or certification is required.

PART II: Information Cited

The undersigned hereby makes of record in the above-identified application the information listed on the attached form PTO-1449 (modified PTO/SB/08). The order of presentation of the references should not be construed as an indication of the importance of the references.

PART III: Remarks

Documents cited anywhere in the Information Disclosure Statement are enclosed unless otherwise indicated. It is respectfully requested that:

1. The Examiner consider completely the cited information, along with any other information, in reaching a determination concerning the patentability of the present claims;

2. The enclosed form PTO-1449 (modified PTO/SB/08) be signed by the Examiner to evidence that the cited information has been fully considered by the United States Patent and Trademark Office during the examination of this application;

3. The citations for the information be printed on any patent which issues from this application.

By submitting this Information Disclosure Statement, the undersigned makes no representation that a search has been performed, of the extent of any search performed, or that more relevant information does not exist.

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, in fact, prior art as defined by 35 U.S.C. § 102.

Notwithstanding any statements by the undersigned, the Examiner is urged to form his or her own conclusion regarding the relevance of the cited information.

An early and favorable action is hereby requested.

The Director is hereby authorized to charge any deficiency or credit any overpayment in the fees occasioned by the filing of this Information Disclosure Statement to our Deposit Account No. 23/2825 under Docket No. M0546.70012US01 from which the undersigned is authorized to draw.

Respectfully submitted,

By: /Amy J. McMahon/
Amy J. McMahon, PhD, Reg. No. 73,073
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
Telephone: (617) 646-8000

Docket No.: M0546.70012US01
Date: July 23, 2018

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 15/988,463	ATTY. DOCKET NO.: M0546.70012US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: May 24, 2018	CONFIRMATION NO.: 7597
				FIRST NAMED INVENTOR: Stuart Alexander Cook	
Sheet	1	of	1	GROUP ART UNIT: 1646	EXAMINER: Prema Maria Mertz Not Yet Assigned

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document		Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
		Number	Kind Code		

FOREIGN PATENT DOCUMENTS

Examiner's Initials #	Cite No.	Foreign Patent Document			Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		Office/ Country	Number	Kind Code			
		EP	1630232	A2	Conaris research institute AG	03-01-2006	
		WO	1996/019574	A1	Genetics Institute, Inc.	06-27-1996	
		WO	1999/020755	A2	Glaxo Group Limited	04-29-1999	
		WO	2005/058956	A1	Commonwealth Scientific and Industrial Research Organisation	06-30-2005	

OTHER ART -- NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	Translation (Y/N)
		CHOW et al., Structure of an extracellular gp130 cytokine receptor signaling complex. Science. 2001 Mar 16;291(5511):2150-5.	
		JOHNSTONE et al., Emerging roles for IL-11 signaling in cancer development and progression: Focus on breast cancer. Cytokine Growth Factor Rev. 2015 Oct;26(5):489-98. doi: 10.1016/j.cytogfr.2015.07.015. Epub 2015 Jul 14.	
		LEMOLI et al., Interleukin-11 (IL-11) acts as a synergistic factor for the proliferation of human myeloid leukaemic cells. Br J Haematol. 1995 Oct;91(2):319-26.	
		[No Author Listed] Recombinant Human Anti-human Il11 Antibody. Creative Biolabs. 2018 May 8.	
		PUTOCZKI et al., Interleukin-11 is the dominant IL-6 family cytokine during gastrointestinal tumorigenesis and can be targeted therapeutically. Cancer Cell. 2013 Aug 12;24(2):257-71. doi: 10.1016/j.ccr.2013.06.017.	
		SOMMER et al., Constitutively active mutant gp130 receptor protein from inflammatory hepatocellular adenoma is inhibited by an anti-gp130 antibody that specifically neutralizes interleukin 11 signaling. J Biol Chem. 2012 Apr 20;287(17):13743-51. doi: 10.1074/jbc.M111.349167.	

[NOTE – No copies of U.S. patents, published U.S. patent applications, or pending, unpublished patent applications stored in the USPTO's Image File Wrapper (IFW) system, are included. See 37 CFR § 1.98 and 1287OG163. Copies of all other patent(s), publication(s), unpublished, pending U.S. patent applications, or other information listed are provided as required by 37 CFR § 1.98 unless 1) such copies were provided in an IDS in an earlier application that complies with 37 CFR § 1.98, and 2) the earlier application is relied upon for an earlier filing date under 35 U.S.C. § 120.]

EXAMINER:	DATE CONSIDERED:

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 630 232 A1

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
01.03.2006 Bulletin 2006/09

(51) Int Cl.:
C12N 15/67 *(2006.01)* ***C07K 14/715*** *(2006.01)*

(21) Application number: **04020455.4**

(22) Date of filing: **27.08.2004**

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LI LU MC NL PL PT RO SE SI SK TR
Designated Extension States:
AL HR LT LV MK

(71) Applicant: **CONARIS research institute AG**
24118 Kiel (DE)

(72) Inventors:
- **Seegert, Dirk**
  24161 Altenholz (DE)
- **Wätzig, Georg**
  24106 Kiel (DE)
- **Rahaus, Nikolaus**
  24159 Kiel (DE)
- **Daecke, Jessica**
  24118 Kiel (DE)
- **Rose-John, Stefan**
  24211 Schellhorn (DE)

(74) Representative: **Schüssler, Andrea**
Kanzlei Huber & Schüssler
Truderinger Strasse 246
81825 München (DE)

Remarks:
The sequence listing, which is published as annex to the application documents, was filed after the date of filing. The applicant has declared that it does not include matter which goes beyond the content of the application as filed.

(54) Optimized nucleotide sequences encoding sgp130

(57) Described are codon optimized sgp130 encoding nucleic acid molecules as well as a method for the highly efficient recombinant production of sgp130 in mammalian cells or bacteria using a nucleic acid molecule of the invention.

EP 1 630 232 A1

Printed by Jouve, 75001 PARIS (FR)

Description

[0001] The present invention relates to codon optimized sgp130 encoding nucleic acid molecules as well as a method for the highly efficient recombinant production of sgp130 in mammalian cells or bacteria using a nucleic acid molecule of the invention.

[0002] For the treatment of various diseases such as Crohn's disease etc. the specific blocking of IL-6 responses dependent on soluble IL-6R might be desirable for treatment. It was found that a soluble gp130-dimer, in particular an IgG-Fc fusion protein or a PEGylated version of sgp130, efficiently inhibits the anti-apoptotic effect of sIL-6R from LPMC from Crohn's disease (CD) patients and that, thus, said compound is useful for the treatment of said disease and related diseases like, e.g., colitis or rheumatoid arthritis. Unfortunately, so far the recombinant production of sgp130 is difficult in particular due to the fact that only low amounts of protein can be obtained

[0003] Thus, the technical problem underlying the present invention was to provide means allowing to improve the efficiency of recombinant production of sgp130Fc or sgp130(D1-D3).

[0004] The solution of the said technical problem is achieved by providing the embodiments characterized in the claims. During the experiments leading to the present invention it was found that by use of particular codon optimized versions of the DNA encoding sgp130Fc the yields of recombinant protein can be increased at least 10- to 20-fold compared to the unmodified version of the DNA. In case of the prokaryotic sgp130(D1-D3) version, the optimization of the DNA led to the reduction of undesired shorter side products.

Brief description of the drawings

[0005]

Figure 1: Schematic presentation of the constructs
Grey shedding marks the parts of the protein which have been optimized. **(A)** Eukaryotic construct comprising a signal peptide, six extracellular gp130 domains and the IgG-Fc part. **(B)** Variations of the sgp130 protein expressed in prokaryotic cells. sgp130 can be expressed with or without N-terminal leader sequence and/or C-terminal Tag for purification purposes.

Figure 2: sgp130(D1-D3) (nucleotide sequence and amino acid sequence) for expression in bacterial cells
An alignment of the nucleotide sequence with optimized codons (sgp130(D1-D3)_opt) vs. the original sequence (sgp130(D1-D3)_wt) is shown.

Figure 3: sgp130Fc (nucleotide sequence and amino acid sequence) for expression in mammalian cells
An alignment of the nucleotide sequence with optimized codons (sgp130Fc_opt) vs. the original sequence (sgp130Fc_wt) is shown.

Figure 4: Detection of sgp130Fc after transient transfection of HEK293 cells (A) or CHO cells (B) with wildtype or optimized (opt) sgp130Fc expression plasmids
The position of sgp130Fc is indicated by arrows (◀). Wildtype and optimized sgp130Fc expression was detected in two independent transfection experiments each. The different sizes of the protein (left panel) result from the leader sequence which has been partially cleaved off after secretion into the medium. The right panel represents the results derived from whole cell extracts from CHO cells (sgp130Fc with leader sequence).

Figure 5: Detection of RNA transcribed from transfected plasmid DNA (sgp130Fc; neomycin resistance gene (NeoR)) by gene-specific RT-PCR
HEK293 cells were transfected with an expression plasmid encoding either wildtype or optimized sgp130Fc. In addition both plasmids encoded a neomycin resistance gene (NeoR). Transfection of the empty vector (mock) or non-transfected cells (control) served as negative controls. β-actin was amplified from total RNA to demonstrate the use of equal amounts of RNA in each single experiment.

Figure 6: Expression of sgp130(D1-D3) in BL21(DE3)pLys bacteria
The cDNA encoding sgp130(D1-D3) was cloned into the expression plasmid pET22b (Invitrogen, Carlsbad, CA, USA) which in addition encodes a leading pelB sequence and a C-terminal His-tag. sgp130(D1-D3) was detected by western blot with a His-specific antibody and marked with an arrow (◀).

[0006] Thus, the present invention relates to a nucleic acid molecule encoding sgp130 comprising the nucleic acid sequence (a) as depicted in Figure 2 (sgp130(D1-3)_opt) or Figure 3 (sgp130Fc_opt) or (b) a fragment or analogue

thereof which maintains the codon usage pattern thereof.

The letter "s" of sgp130 means "soluble". The term "soluble" as used herein refers to a gp130 molecule lacking the intracellular domain and the transmembrane domain. The domains utilized in sgp130(D1-D3)_opt consist of the first three extracellular domains D1-D3 of gp130.

[0007] The term "fragment" as used herein refers to sgp130 fragments which comprise the entire or smaller parts of the optimized cDNA encoding the extracellular domain of gp130. Preferably, such fragments show a biological activity of the full length molecule, e. g. maintain the ability to inhibit the activity of the agonistic complex IL-6/sIL-6R. For the expression in bacteria such fragment also comprises sgp130 without the eukaryotic secretory leader sequence (MLTLQTWWQALFIFLTTESTG, Pos. 1 to 22). Moreover, a prokaryotic secretory leader sequence, e. g. pelB or *OmpA* could be cloned in front of the sgp130 sequence or parts of it and could be derived from the respective suitable expression plasmid, e. g. pET22b (Merck Biosciences GmbH, Bad Soden, Germany), pASK-IBA2, pASK-IBA12 (IBA, Goettingen, Germany). In addition the sgp130 protein can be expressed with or without Tag for purification purposes, e. g. His_6, Strep, Myc or others.

[0008] The term "analogue" as used herein refers to a nucleic acid molecule which encodes the same amino acid sequence but which, through the redundancy of the genetic code, has a different nucleotide sequence. The term "codon usage pattern" as used herein refers to the average frequencies in the nucleotide sequence, e.g., highly expressed mammalian genes. Codon usage patterns for mammals, including humans can be found in the literature; see, e.g., Nakamura et al., Nucleic Acids Research 1996, 24:214-5. In the nucleic acid molecules of the present invention, the codon usage pattern is altered to more closely represent the codon bias of the host organism, e.g. a mammalian cell.

[0009] Alternatively, the present invention relates to a nucleic acid molecule, wherein at least 80%, preferably at least 90%, more preferably at least 95% and, most preferably at least 98% of the codons altered in the nucleic acid sequence of Figure 2 or 3 vs. the wild type sequence are present.

[0010] In a preferred embodiment, the nucleic acid molecule of the present invention is a DNA molecule.

[0011] The present invention includes expression vectors that comprise the nucleic acid molecules of the invention. The expression vectors can be constructed according to methods well known to the person skilled in the art; see, e.g., Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. The "control elements" or "regulatory sequences" used for recombinant expression are those non-translated regions of the vector-enhancers, promoters, 5' and 3' untranslated regions which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilised, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. Promoters and other expression regulation signals may be selected to be compatible with the host cell for which expression is designed. For example, mammalian promoters include the metallothionein promoter, which can be induced in response to heavy metals such as cadmium, and the β-actin 30 promoter. Viral promoters such as the SV40 large T antigen promoter, human cytomegalovirus (CMV) immediate early (IE) promoter, rous sarcoma virus LTR promoter, adenovirus promoter, or a HPV promoter, particularly the HPV upstream regulatory region (URR) may also be used. All these promoters are well described and readily available in the art.

[0012] In mammalian host cells, a number of viral-based expression systems may be utilised. In cases where an adenovirus is used as an expression vector, sequences encoding the polypeptide(s) of the present invention may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain a viable virus which is capable of expressing the antibody in infected host cells (Logan, J. and Shenk, T. (1984) Proc. Natl. Acad. Sci. 81:3655-3659). In addition, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

[0013] Further examples of suitable viral vectors, include herpes simplex viral vectors, vaccinia or alpha-virus vectors and retroviruses, including lentiviruses and adeno-associated viruses. Gene transfer techniques using these viruses are known to those skilled in the art. Retrovirus vectors for example may be used to stably integrate the nucleic acid molecules of the invention into the host genome, although such recombination is not preferred. Replication-defective adenovirus vectors by contrast remain episomal and therefore allow transient expression. If it is necessary to generate a cell line that contains multiple copies of the sequence encoding the spg130 polypeptides, vectors based on SV40 or EBV may be used with an appropriate selectable marker.

[0014] Human artificial chromosomes (HACs) may also be employed to deliver larger fragments of DNA than can be contained and expressed in a plasmid. HACs of 6 to 10 M are constructed and delivered via conventional delivery methods (liposomes, polycationic amino polymers, or vesicles) for therapeutic purposes.

[0015] Specific initiation signals may also be used to achieve more efficient translation. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding the sgp130, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in case where only a coding sequence for a fragment is inserted, exogenous

translational control signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used, such as those described in the literature (Scharf, D. et al. (1994) Results Probl. Cell Differ. 20:125-162).

[0016] In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed polypeptide chains in the desired fashion. Post-translational processing which cleaves a "prepro" form of the polypeptide may also be used to facilitate correct insertion, folding and/or function. Different mammalian host cells which have specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, 293, COS-7 and W138), are available from the American Type Culture Collection (ATCC; Bethesda, Md.) and may be chosen to ensure the correct modification and processing of the foreign polypeptide chains.

[0017] For long-term, high-yield production of sgp130, stable expression in mammalian cells is preferred. For example, cell lines which stably express sgp130Fc may be transfected using expression vectors which may contain viral origins of replication and/or endogenous expression elements and one or more selectable marker genes on the same or on a separate vector. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be proliferated using tissue culture techniques appropriate to the cell type.

[0018] After the introduction of the recombinant vector(s), the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler, M. et al. (1977) Cell 11: 223-32) and adenine phosphoribosyltransferase (Lowy, I. et al. (1980) Cell 22:817-23) genes which can be employed in tk.sup.- or aprt.sup.- cells, respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection; for example, dhfr which confers resistance to methotrexate (Wigler, M. et al. (1980) Proc. Natl. Acad. Sci. 77:3567-70); npt, which confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin, F. et al (1981) J. Mol. Biol. 150:1-14) and als or pat, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry, supra). Additional selectable genes have been described, for example, trpB, which allows cells to utilise indole in place of tryptophan, or hisD, which allows cells to utilise histinol in place of histidine (Hartman, S. C. and R. C. Mulligan (1988) Proc. Natl. Acad. Sci. 85:8047-51). Recently, the use of visible markers has gained popularity with such markers as anthocyanins, beta-glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes, C. A. et al. (1995) Methods Mol. Biol. 55:121-131).

[0019] The person skilled also knows vectors and host cells for bacterial expression, e.g. bacteriophage, plasmid, or cosmid DNA expression vectors. Vectors suitable for use in the present invention include, but are not limited to the pSKK expression vector for expression in bacteria. Depending on the vector system and host utilised, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid *lacZ* promoter of the Bluescript.RTM. phagemid (Stratagene, LaJolla, Calif.) or pSport1.TM. plasmid (Gibco BRL) and the like may be used.

[0020] Purification of the recombinant sgp130 is carried out by any one of the methods known for this purpose, i.e., any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like. A further purification procedure that may be used is affinity chromatography using monoclonal antibodies which bind the target polypeptide or a Tag fused to it, e.g., His, Strep or Myc, and which are produced and immobilized on a gel matrix contained within a column. Impure preparations containing the recombinant sgp130 are passed through the column. The sgp130 will be bound to the column by the specific antibody while the impurities will pass through. After washing the polypeptide is eluted from the gel by a change in pH or ionic strength and can then, if desired, dimerized and/or PEGylated.

[0021] Accordingly, the present invention also relates to a method of producing the sgp130 of the present invention, comprising culturing a host cell transformed with a nucleic acid molecule of the invention and recovering the sgp130 polypeptide from said host cell or the culture.

[0022] The sgp130 polypeptide produced from a nucleic acid molecule of the present invention is useful in the treatment and/or prevention of all the pathologies, in which the activity of the agonistic complex IL-6/sIL6R must be inhibited, e.g., for the treatment/prevention of bone resorption, hypercalcemia, cachexia, tumours (such as colon cancer), autoimmune diseases (e.g. systemic lupus erythematosis, SLE), chronic inflammations (such as Crohn's disease or rheumatoid arthritis) and bacterial or viral infections.

[0023] The below examples explain the invention in more detail.

Example 1: Material and Methods

(A) Construct and transfection

[0024] The cDNA encoding either wildtype or optimized sgp130Fc was cloned into the expression plasmid pDEST40 (Invitrogen, Carlsbad, CA, USA) according to standard procedures. The wildtype sequence was derived from an expression plasmid which has been described in Jostock et al., Eur. J. Biochem. 268 (2001), 160-7 (Figure 1; upper panel). The construct was sequence verified. 3 x 10^5 HEK293 cells were transiently transfected with 1 μg of plasmid and 3 μl of Fugene (Roche Diagnostics, Mannheim, Germany) in 3 ml of medium according to the manufacturer's manual. The cells were subsequently incubated for 24 h at 37°C and supernatants and cells were harvested for further preparations of either total proteins or RNA, respectively.

[0025] One set of cells was transfected with the empty vector (mock), another set of cells was left untransfected (control). Both sets served as negative controls.

(B) Protein extraction and western blot

[0026] The sgp130Fc protein was precipitated from the cell supernatants by adding 20 μl of Protein-A/G-Plus Agarose (Santa Cruz, CA, USA). The slurry was incubated overnight at 4°C and finally centrifuged. Bound proteins were extracted by boiling the agarose pellet in SDS sample buffer for 5 minutes at 100°C. In parallel the cells were scraped from the plates using a rubber policeman, harvested in 100 μl of PBS and centrifuged. All protein samples were separated on a standard acrylamide gel, transferred to a PDVF-membrane by semi-dry blotting and stained with a gp130-specific antibody (Hölzel Diagnostika, Köln, Germany). 50 ng of recombinant sgp130 served as positive control (sgp130).

(C) RNA extraction

[0027] Total RNA was extracted from the cell pellets using a RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The following primers were used to determine RNA transcribed from the transfected plasmid DNA by RT-PCR: sgp130Fc_f: 5'-ATGAGGTGTGAGTGGGATGG-3', sgp130Fc_r: 5'-ACCTTGCACTTGTACTCCTTGC-3'; neomycin resistance gene NeoR_s: 5'-GATGCCTGCTTGCCGAATATC-3'; NeoR_r: 5'-CGCCAAGCTCTTCAGCAATATC-3'. Total RNA was initially reverse transcribed and the cDNA was amplified by 30 cycles of 30 seconds at 95°C followed by 2 minutes at 57°C and a final elongation step of 5 minutes at 72°C. Expected amplicon sizes: gp130: 1.712 bp, NeoR: 133 bp. The amplification of NeoR was performed to document an equal transfection efficiency of the plasmid. In addition, β-actin was amplified to demonstrated the use of equal amounts of total RNA in each experiment.

Example 2: Highly efficient recombinant production of sgp130Fc in HEK293 cells

[0028] Figure 4 demonstrates that in comparison to the wildtype expression plasmid the production of sgp130Fc was increased at least 10 to 20-fold in HEK293 cells transfected with the optimized sgp130Fc expression plasmid. On the RNA level (Figure 5) a similar increase of sgp130Fc expression was detected with the optimized construct. This elevation of sgp130Fc RNA amounts was not due to a different transfection efficiency as shown by equal amounts of RNA encoded by the neomycin resistance gene which was also located on the expression plasmid.

[0029] The results indicate that the significant increase of sgp130Fc production after optimization of the cDNA sequence is partially based on an improved codon usage during translation but is mainly derived from the elevation of the corresponding RNA levels. This might be due to a more efficient transcription or a higher stability of the RNA.

Example 3: Highly efficient recombinant production of sgp130(D1-D3) in bacteria

(A) Constructs and transformation

[0030] The cDNA encoding either wildtype or optimized sgp130(D1-D3) was cloned into the prokaryotic expression plasmid pET22b (Merck Biosciences GmbH, Bad Soden, Germany) according to standard procedures. The D1-D3 fragment was amplified afore from the pSVL-sgp130Fc plasmid described in Jostock et al. 2001. The construct was sequence verified and transformed into BL21(DE3)pLys bacteria (Invitrogen, Carlsbad, CA, USA).

(B) Protein expression and western blot

[0031] 10 ml of bacterial suspension were diluted at 1:100 with LB-medium and grown at 30°C overnight until the $OD_{600\,nm}$ reached a value of 0.3 (250 rpm). Protein expression was induced by the addition of 0.3 mM of IPTG (Isopro-

pyl-beta-D-thiogalactopyranoside) (Qiagen, Hilden, Germany) and further incubation of the cells overnight at 25°C. The cells were pelleted by centrifugation at 4°C and 4600 rpm for 30 minutes and the pellet was resuspended in 1 ml PBS (PAA Laboratories GmbH, Cölbe, Germany). Disruption of the cells was performed by sonication (3 x 30 sec, 10% cycle, 20% power) with a Bandelin Sonoplus HD 2070 sonicator. Insoluble material was pelleted at 13.000 rpm and 4°C for 30 min and the pellet was resuspended in 1 ml of urea buffer (50 mM NaH_2PO_4, 8 M urea, pH8). An aliquot was diluted at 1:100 and subsequently analyzed by SDS PAGE according to standard protocols. His-tagged target proteins were detected with an anti-PentaHis antibody (Qiagen, Hilden, Germany).

(C) Results

[0032] Whereas the wildtype sequence generated a second shorter form of sgp130(D1-D3) (Figure 6, left lane) this by-product was not observed with the optimized cDNA (Figure 6, right lane). This unwanted variation of sgp130(D1-D3) is generated by further alternative transcriptional and translational start sides which have been eliminated by codon modifications in the optimized cDNA sequence. Subsequently the efficiency to produce the desired protein with the right size was increased at least at a factor of 3-fold.

SEQUENCE LISTING

<110> Conaris research institute AG

<120> Optimized nucleotide sequences encoding sgp130

<130> C 1028EP

<140> EP 04020455.4
<141> 2004-08-27

<160> 13

<170> PatentIn version 3.2

<210> 1
<211> 22
<212> PRT
<213> Artificial sequence

<220>
<223> secretory leader sequence of sgp130

<400> 1

Met Leu Thr Leu Gln Thr Trp Val Val Gln Ala Leu Phe Ile Phe Leu
1 5 10 15

Thr Thr Glu Ser Thr Gly
20

<210> 2
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<223> Primer

<400> 2
atgaggtgtg agtgggatgg 20

<210> 3
<211> 22
<212> DNA
<213> Artificial sequence

<220>
<223> Primer

<400> 3
accttgcact tgtactcctt gc 22

<210> 4
<211> 21
<212> DNA
<213> Artificial sequence

<220>
<223> Primer

<400> 4
gatgcctgct tgccgaatat c 21

<210> 5
<211> 22
<212> DNA
<213> Artificial sequence

<220>
<223> Primer

<400> 5
cgccaagctc ttcagcaata tc 22

<210> 6
<211> 918
<212> DNA
<213> sgp130(d1-D3)_wt

<400> 6
atggaactgc tggacccgtg cggttatatc agcccggaaa gcccagttgt tcaactgcat 60
agcaatttca ccgcggtttg tgtgctgaaa gagaagtgta tggattattt ccacgtcaac 120
gcgaactaca ttgtgtggaa aaccaaccat ttcaccatcc cgaaagaaca gtataccatc 180
attaaccgta ccgcgagcag cgttaccttt accgatatcg cgagcctgaa cattcaactg 240
acctgcaaca ttctgacctt cggtcaactg gaacagaatg tttatggcat caccatcatt 300
agcggcctgc cgccggaaaa accgaaaaat ctgagctgca ttgtgaacga aggcaaaaaa 360
atgcgctgcg aatgggatgg tggtcgcgaa acccacctgg aaaccaactt caccctgaaa 420
agcgaatggg cgacccacaa atttgcggat tgcaaagcga aacgcgatac cccgacctct 480
tgcaccgttg attacagcac cgtgtacttc gtgaacattg aagtgtgggt ggaagcggaa 540
aacgccctgg gtaaagtcac cagcgatcat atcaactttg atccggtgta caaagtgaaa 600
ccgaatccgc cgcataatct gagcgtgatc aacagcgaag aactgagcag catcctgaaa 660
ctgacctgga ccaacccgag cattaagagc gttatcatcc tgaaatacaa cattcagtat 720
cgcaccaaag atgccagcac ctggagccag attccgccgg aagataccgc gagcacccgt 780
agcagcttca ccgttcagga tctgaaaccg tttaccgaat atgtgtttcg cattcgctgt 840
atgaaagaag atggtaaagg ctactggagc gattggagcg aagaagcgag cggtatcacc 900
tatgaagatc gtccgtga 918

<210> 7
<211> 918
<212> DNA
<213> Artificial sequence

<220>
<223> sgp130(D1-D3)_opt

<400> 7
atggaacttc tagatccatg tggttatatc agtcctgaat ctccagttgt acaacttcat 60
tctaatttca ctgcagtttg tgtgctaaag gaaaaatgta tggattattt tcatgtaaat 120
gctaattaca ttgtctggaa aacaaaccat tttactattc ctaaggagca atatactatc 180
ataaacagaa cagcatccag tgtcaccttt acagatatag cttcattaaa tattcagctc 240

acttgcaaca ttcttacatt cggacagctt gaacagaatg tttatggaat cacaataatt 300

tcgggcttgc ctccagaaaa acctaaaaat ttgagttgca ttgtgaacga ggggaagaaa 360

atgaggtgtg agtgggatgg tggaagggaa acacacttgg agacaaactt cactttaaaa 420

tctgaatggg caacacacaa gtttgctgat tgcaaagcaa aacgtgacac ccccacctca 480

tgcactgttg attattctac tgtgtatttt gtcaacattg aagtctgggt agaagcagag 540

aatgcccttg ggaaggttac atcagatcat atcaattttg atcctgtata taaagtgaag 600

cccaatccgc cacataattt atcagtgatc aactcagagg aactgtctag tatcttaaaa 660

ttgacatgga ccaacccaag tattaagagt gttataatac taaaatataa cattcaatat 720

aggaccaaag atgcctcaac ttggagccag attcctcctg aagacacagc atccacccga 780

tcttcattca ctgtccaaga ccttaaacct tttacagaat atgtgtttag gattcgctgt 840

atgaaggaag atggtaaggg atactggagt gactggagtg aagaagcaag tgggatcacc 900

tatgaagata gaccatga 918

<210> 8
<211> 305
<212> PRT
<213> Artificial sequence

<220>
<223> sgp130(D1-D3)_wt

<400> 8

Met Glu Leu Leu Asp Pro Cys Gly Tyr Ile Ser Pro Glu Ser Pro Val
1 5 10 15

Val Gln Leu His Ser Asn Phe Thr Ala Val Cys Val Leu Lys Glu Lys
20 25 30

Cys Met Asp Tyr Phe His Val Asn Ala Asn Tyr Ile Val Trp Lys Thr
35 40 45

Asn His Phe Thr Ile Pro Lys Glu Gln Tyr Thr Ile Ile Asn Arg Thr
50 55 60

Ala Ser Ser Val Thr Phe Thr Asp Ile Ala Ser Leu Asn Ile Gln Leu
65 70 75 80

Thr Cys Asn Ile Leu Thr Phe Gly Gln Leu Glu Gln Asn Val Tyr Gly
85 90 95

Ile Thr Ile Ile Ser Gly Leu Pro Pro Glu Lys Pro Lys Asn Leu Ser
100 105 110

Cys Ile Val Asn Glu Gly Lys Lys Met Arg Cys Glu Trp Asp Gly Gly
115 120 125

```
Arg Glu Thr His Leu Glu Thr Asn Phe Thr Leu Lys Ser Glu Trp Ala
    130                 135                 140

Thr His Lys Phe Ala Asp Cys Lys Ala Lys Arg Asp Thr Pro Thr Ser
145                 150                 155                 160

Cys Thr Val Asp Tyr Ser Thr Val Tyr Phe Val Asn Ile Glu Val Trp
                165                 170                 175

Val Glu Ala Glu Asn Ala Leu Gly Lys Val Thr Ser Asp His Ile Asn
            180                 185                 190

Phe Asp Pro Val Tyr Lys Val Lys Pro Asn Pro Pro His Asn Leu Ser
        195                 200                 205

Val Ile Asn Ser Glu Glu Leu Ser Ser Ile Leu Lys Leu Thr Trp Thr
    210                 215                 220

Asn Pro Ser Ile Lys Ser Val Ile Ile Leu Lys Tyr Asn Ile Gln Tyr
225                 230                 235                 240

Arg Thr Lys Asp Ala Ser Thr Trp Ser Gln Ile Pro Pro Glu Asp Thr
                245                 250                 255

Ala Ser Thr Arg Ser Ser Phe Thr Val Gln Asp Leu Lys Pro Phe Thr
            260                 265                 270

Glu Tyr Val Phe Arg Ile Arg Cys Met Lys Glu Asp Gly Lys Gly Tyr
        275                 280                 285

Trp Ser Asp Trp Ser Glu Glu Ala Ser Gly Ile Thr Tyr Glu Asp Arg
    290                 295                 300

Pro
305
```

<210> 9
<211> 305
<212> PRT
<213> Artificial sequence

<220>
<223> sgp130(D-D3)_opt

<400> 9

```
Met Glu Leu Leu Asp Pro Cys Gly Tyr Ile Ser Pro Glu Ser Pro Val
1               5                   10                  15

Val Gln Leu His Ser Asn Phe Thr Ala Val Cys Val Leu Lys Glu Lys
            20                  25                  30

Cys Met Asp Tyr Phe His Val Asn Ala Asn Tyr Ile Val Trp Lys Thr
        35                  40                  45
```

```
Asn His Phe Thr Ile Pro Lys Glu Gln Tyr Thr Ile Ile Asn Arg Thr
    50                  55                  60

Ala Ser Ser Val Thr Phe Thr Asp Ile Ala Ser Leu Asn Ile Gln Leu
65                  70                  75                  80

Thr Cys Asn Ile Leu Thr Phe Gly Gln Leu Glu Gln Asn Val Tyr Gly
                85                  90                  95

Ile Thr Ile Ile Ser Gly Leu Pro Pro Glu Lys Pro Lys Asn Leu Ser
            100                 105                 110

Cys Ile Val Asn Glu Gly Lys Lys Met Arg Cys Glu Trp Asp Gly Gly
        115                 120                 125

Arg Glu Thr His Leu Glu Thr Asn Phe Thr Leu Lys Ser Glu Trp Ala
    130                 135                 140

Thr His Lys Phe Ala Asp Cys Lys Ala Lys Arg Asp Thr Pro Thr Ser
145                 150                 155                 160

Cys Thr Val Asp Tyr Ser Thr Val Tyr Phe Val Asn Ile Glu Val Trp
                165                 170                 175

Val Glu Ala Glu Asn Ala Leu Gly Lys Val Thr Ser Asp His Ile Asn
            180                 185                 190

Phe Asp Pro Val Tyr Lys Val Lys Pro Asn Pro Pro His Asn Leu Ser
        195                 200                 205

Val Ile Asn Ser Glu Glu Leu Ser Ser Ile Leu Lys Leu Thr Trp Thr
    210                 215                 220

Asn Pro Ser Ile Lys Ser Val Ile Ile Leu Lys Tyr Asn Ile Gln Tyr
225                 230                 235                 240

Arg Thr Lys Asp Ala Ser Thr Trp Ser Gln Ile Pro Pro Glu Asp Thr
                245                 250                 255

Ala Ser Thr Arg Ser Ser Phe Thr Val Gln Asp Leu Lys Pro Phe Thr
            260                 265                 270

Glu Tyr Val Phe Arg Ile Arg Cys Met Lys Glu Asp Gly Lys Gly Tyr
        275                 280                 285

Trp Ser Asp Trp Ser Glu Glu Ala Ser Gly Ile Thr Tyr Glu Asp Arg
    290                 295                 300

Pro
305
```

<210> 10
<211> 2511
<212> DNA
<213> artificial

<220>
<223> sgp130Fc_wt

<400> 10
atgttgacgt tgcagacttg gctagtgcaa gccttgttta ttttcctcac cactgaatct 60
acaggtgaac ttctagatcc atgtggttat atcagtcctg aatctccagt tgtacaactt 120
cattctaatt tcactgcagt ttgtgtgcta aaggaaaaat gtatggatta ttttcatgta 180
aatgctaatt acattgtctg gaaaacaaac cattttacta ttcctaagga gcaatatact 240
atcataaaca gaacagcatc cagtgtcacc tttacagata tagcttcatt aaatattcag 300
ctcacttgca acattcttac attcggacag cttgaacaga atgtttatgg aatcacaata 360
atttcgggct tgcctccaga aaaacctaaa aatttgagtt gcattgtgaa cgaggggaag 420
aaaatgaggt gtgagtggga tggtggaagg gaaacacact tggagacaaa cttcacttta 480
aaatctgaat gggcaacaca caagtttgct gattgcaaag caaaacgtga cacccccacc 540
tcatgcactg ttgattattc tactgtgtat tttgtcaaca ttgaagtctg ggtagaagca 600
gagaatgccc ttgggaaggt tacatcagat catatcaatt ttgatcctgt atataaagtg 660
aagcccaatc cgccacataa tttatcagtg atcaactcag aggaactgtc tagtatctta 720
aaattgacat ggaccaaccc aagtattaag agtgttataa tactaaaata taacattcaa 780
tataggacca aagatgcctc aacttggagc cagattcctc ctgaagacac agcatccacc 840
cgatcttcat tcactgtcca agaccttaaa ccttttacag aatatgtgtt taggattcgc 900
tgtatgaagg aagatggtaa gggatactgg agtgactgga gtgaagaagc aagtgggatc 960
acctatgaag atagaccatc taaagcacca agtttctggt ataaaataga tccatcccat 1020
actcaaggct acagaactgt acaactcgtg tggaagacat tgcctccttt tgaagccaat 1080
ggaaaaatct tggattatga agtgactctc acaagatgga aatcacattt acaaaattac 1140
acagttaatg ccacaaaact gacagtaaat ctcacaaatg atcgctatct agcaacccta 1200
acagtaagaa atcttgttgg caaatcagat gcagctgttt taactatccc tgcctgtgac 1260
tttcaagcta ctcaccctgt aatggatctt aaagcattcc ccaaagataa catgctttgg 1320
gtggaatgga ctactccaag ggaatctgta aagaaatata tacttgagtg gtgtgtgtta 1380
tcagataaag caccctgtat cacagactgg caacaagaag atggtaccgt gcatcgcacc 1440
tatttaagag ggaacttagc agagagcaaa tgctatttga taacagttac tccagtatat 1500
gctgatggac caggaagccc tgaatccata aaggcatacc ttaaacaagc tccaccttcc 1560
aaaggaccta ctgttcggac aaaaaaagta gggaaaaacg aagctgtctt agagtgggac 1620
caacttcctg ttgatgttca gaatggattt atcagaaatt atactatatt ttatagaacc 1680
atcattggaa atgaaactgc tgtgaatgtg gattcttccc acacagaata tacattgtcc 1740

tctttgacta gtgacacatt gtacatggta cgaatggcag catacacaga tgaaggtggg 1800

aaggatggtc cagaattcag atcttgtgac aaaactcaca catgcccacc gtgcccagca 1860

cctgaagccg agggcgcgcc gtcagtcttc ctcttccccc caaaacccaa ggacaccctc 1920

atgatctccc ggaccctga ggtcacatgc gtggtggtgg acgtgagcca cgaagaccct 1980

gaggtcaagt tcaactggta cgtggacggc gtggaggtgc ataatgccaa gacaaagccg 2040

cgggaggagc agtacaacag cacgtaccgt gtggtcagcg tcctcaccgt cctgcaccag 2100

gactggctga atggcaagga gtacaagtgc aaggtctcca acaaagccct cccagccccc 2160

atcgagaaaa ccatctccaa agccaaaggg cagccccgag aaccacaggt gtacaccctg 2220

cccccatccc gggaggagat gaccaagaac caggtcagcc tgacctgcct ggtcaaaggc 2280

ttctatccca gcgacatcgc cgtggagtgg gagagcaatg ggcagccgga gaacaactac 2340

aagaccacgc ctcccgtgct ggactccgac ggctccttct tcctctatag caagctcacc 2400

gtggacaaga gcaggtggca gcaggggaac gtcttctcat gctccgtgat gcatgaggct 2460

ctgcacaacc actacacgca gaagagcctc tccctgtctc cgggtaaatg a 2511

<210> 11
<211> 835
<212> PRT
<213> artificial

<220>
<223> sgp130Fc_wt

<400> 11

```
Met Leu Thr Leu Gln Thr Trp Leu Val Gln Ala Leu Phe Ile Phe Leu
1               5                  10                  15

Thr Thr Glu Ser Thr Gly Glu Leu Leu Asp Pro Cys Gly Tyr Ile Ser
            20                  25                  30

Pro Glu Ser Pro Val Val Gln Leu His Ser Asn Phe Thr Ala Val Cys
        35                  40                  45

Val Leu Lys Glu Lys Cys Met Asp Tyr Phe His Val Asn Ala Asn Tyr
    50                  55                  60

Ile Val Trp Lys Thr Asn His Phe Thr Ile Pro Lys Glu Gln Tyr Thr
65                  70                  75                  80

Ile Ile Asn Arg Thr Ala Ser Ser Val Thr Phe Thr Asp Ile Ala Ser
                85                  90                  95

Leu Asn Ile Gln Leu Thr Cys Asn Ile Leu Thr Phe Gly Gln Leu Glu
            100                 105                 110

Gln Asn Tyr Tyr Gly Ile Thr Ile Ile Ser Gly Leu Pro Pro Glu Lys
        115                 120                 125
```

```
Pro Lys Asn Leu Ser Cys Ile Val Asn Glu Gly Lys Lys Met Arg Cys
    130                 135                 140

Glu Trp Asp Gly Gly Arg Glu Thr His Leu Glu Thr Asn Phe Thr Leu
145                 150                 155                 160

Lys Ser Glu Trp Ala Thr His Lys Phe Ala Asp Cys Lys Ala Lys Arg
                165                 170                 175

Asp Thr Pro Thr Ser Cys Thr Val Asp Tyr Ser Thr Val Tyr Phe Val
            180                 185                 190

Asn Ile Glu Val Trp Val Glu Ala Glu Asn Ala Leu Gly Lys Val Thr
        195                 200                 205

Ser Asp His Ile Asn Phe Asp Pro Val Tyr Lys Val Lys Pro Asn Pro
    210                 215                 220

Pro His Asn Leu Ser Val Ile Asn Ser Glu Glu Leu Ser Ser Ile Leu
225                 230                 235                 240

Lys Leu Thr Trp Thr Asn Pro Ser Ile Lys Ser Val Ile Ile Leu Lys
                245                 250                 255

Tyr Asn Ile Gln Tyr Arg Thr Lys Asp Ala Ser Thr Trp Ser Gln Ile
            260                 265                 270

Pro Pro Glu Asp Thr Ala Ser Thr Arg Ser Ser Phe Thr Val Gln Asp
        275                 280                 285

Leu Lys Pro Phe Thr Glu Tyr Val Phe Arg Ile Arg Cys Met Lys Glu
    290                 295                 300

Asp Gly Lys Gly Val Trp Ser Asp Trp Ser Glu Glu Ala Ser Gly Ile
305                 310                 315                 320

Thr Tyr Glu Asp Arg Pro Ser Lys Ala Pro Ser Phe Trp Tyr Lys Ile
                325                 330                 335

Asp Pro Ser His Thr Gln Gly Tyr Arg Thr Val Gln Leu Val Trp Lys
            340                 345                 350

Thr Leu Pro Pro Phe Glu Ala Asn Gly Lys Ile Leu Asp Tyr Glu Val
        355                 360                 365

Thr Leu Thr Arg Trp Lys Ser His Leu Gln Asn Tyr Thr Val Asn Ala
    370                 375                 380

Thr Lys Leu Thr Val Asn Leu Thr Asn Asp Arg Tyr Leu Ala Thr Leu
385                 390                 395                 400
```

```
Thr Val Arg Asn Leu Val Gly Lys Ser Asp Ala Ala Val Leu Thr Ile
                405                 410                 415

Pro Ala Cys Asp Phe Gln Ala Thr His Pro Val Met Asp Leu Lys Ala
            420                 425                 430

Phe Pro Lys Asp Asn Met Leu Trp Val Glu Trp Thr Thr Pro Arg Glu
        435                 440                 445

Ser Val Lys Lys Tyr Ile Leu Glu Trp Cys Val Leu Ser Asp Lys Ala
    450                 455                 460

Pro Cys Ile Thr Asp Trp Gln Gln Glu Asp Gly Thr Val His Arg Thr
465                 470                 475                 480

Tyr Leu Arg Gly Asn Leu Ala Glu Ser Lys Cys Tyr Leu Ile Thr Val
                485                 490                 495

Thr Pro Val Tyr Ala Asp Gly Pro Gly Ser Pro Glu Ser Ile Lys Ala
            500                 505                 510

Tyr Leu Lys Gln Ala Pro Pro Ser Lys Gly Pro Thr Val Arg Thr Lys
        515                 520                 525

Lys Val Gly Lys Asn Glu Ala Val Leu Glu Trp Asp Gln Leu Pro Val
    530                 535                 540

Asp Val Gln Asn Gly Phe Ile Arg Asn Tyr Thr Ile Phe Tyr Arg Thr
545                 550                 555                 560

Ile Ile Gly Asn Glu Thr Ala Val Asn Val Asp Ser Ser His Thr Glu
                565                 570                 575

Tyr Thr Leu Ser Ser Leu Thr Ser Asp Thr Leu Tyr Met Val Arg Met
            580                 585                 590

Ala Ala Tyr Thr Asp Glu Gly Gly Lys Asp Gly Pro Glu Phe Arg Ser
        595                 600                 605

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Ala Glu
    610                 615                 620

Gly Ala Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
625                 630                 635                 640

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
                645                 650                 655

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
            660                 665                 670
```

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
675 680 685

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
690 695 700

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
705 710 715 720

Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
725 730 735

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
740 745 750

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
755 760 765

Glu Trp Glu Ser Asn Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro
770 775 780

Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val
785 790 795 800

Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
805 810 815

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser
820 825 830

Pro Gly Lys
835

<210> 12
<211> 2511
<212> DNA
<213> artificial

<220>
<223> sgp130Fc_opt

<400> 12
atgctgacac tgcagacatg gctggtgcag gccctgttta tctttctgac caccgagtct 60

acaggagagc tgctggatcc ttgcggctat atctcccctg agtctcctgt ggtgcagctg 120

cattctaact tcaccgccgt gtgtgtgctg aaggaaaagt gcatggacta cttccacgtg 180

aacgccaact acatcgtgtg gaaaaccaac cacttcacca tccccaagga gcagtacacc 240

atcatcaacc ggaccgcttc ttctgtgacc ttcaccgata tcgcctccct gaatatccag 300

ctgacctgca acatcctgac ctttggacag ctggagcaga atgtgtacgg catcaccatc 360

atctctggcc tgcctccaga gaagcctaag aacctgtcct gcatcgtgaa tgagggcaag 420

aagatgaggt gtgagtggga tggcggcaga gagacacatc tggagaccaa cttcaccctg 480
aagtctgagt gggccaccca caagtttgcc gactgcaagg ccaagagaga tacccctacc 540
tcttgcaccg tggactactc caccgtgtac ttcgtgaaca tcgaggtgtg ggtggaggct 600
gagaatgctc tgggcaaggt gacctctgac cacatcaact tcgaccccgt gtacaaggtg 660
aagcctaacc ctcctcacaa cctgtccgtg atcaactctg aggagctgtc ctctatcctg 720
aagctgacct ggaccaaccc ttccatcaag tccgtgatca tcctgaagta caacatccag 780
tacaggacca aggatgcttc tacctggtct cagatccctc ctgaggatac cgcttccacc 840
agatccagct tcacagtgca ggacctgaag ccttttaccg agtacgtgtt caggatccgg 900
tgcatgaagg aggatggcaa gggctattgg tctgactggt ctgaggaggc ttctggcatc 960
acctacgagg acagaccttc taaggccccc agcttctggt acaagatcga cccttctcac 1020
acccagggct atagaacagt gcagctggtg tggaaaaccc tgcctccatt cgaggctaat 1080
ggcaagatcc tggactatga ggtgaccctg accagatgga agtctcacct gcagaactac 1140
accgtgaacg ctaccaagct gaccgtgaac ctgaccaacg atagatacct ggctaccctg 1200
accgtgagaa atctggtggg caagtctgat gctgctgtgc tgaccatccc tgcctgtgat 1260
tttcaggcta cccaccctgt gatggatctg aaggccttcc ccaaggataa catgctgtgg 1320
gtggagtgga caacacctag agagtccgtg aagaagtaca tcctggagtg gtgcgtgctg 1380
tctgataagg ccccttgcat cacagattgg cagcaggagg atggcaccgt gcatagaacc 1440
tacctgagag gcaatctggc cgagtctaag tgctatctga tcaccgtgac ccctgtgtat 1500
gctgatggac ctggctctcc tgagtctatc aaggcctacc tgaagcaggc tcctccatct 1560
aagggaccta ccgtgaggac aaagaaggtg ggcaagaacg aggctgtgct ggagtgggat 1620
cagctgcctg tggatgtgca gaacggcttc atccggaact acaccatctt ctaccggacc 1680
atcatcggca atgagaccgc cgtgaacgtg gattcttccc acaccgagta cacactgtcc 1740
tctctgacct ctgacaccct gtacatggtg agaatggccg cttataccga tgagggcggc 1800
aaggatggac ctgagttcag atcctgcgac aagacccaca cctgtcctcc ttgtcctgct 1860
cctgaggctg agggcgctcc ttctgtgttt ctgttccccc caaagcctaa ggataccctg 1920
atgatctcca gaacccctga ggtgacatgt gtggtggtgg atgtgtctca tgaggacccc 1980
gaggtgaagt tcaactggta cgtggatggc gtggaggtgc acaatgctaa gaccaagcct 2040
agggaggagc agtacaactc cacctacaga gtggtgtctg tgctgacagt gctgcatcag 2100
gattggctga acggcaagga gtacaagtgc aaggtgtcca acaaggctct gcctgctcct 2160
atcgaaaaga ccatctccaa ggctaaggga cagcctagag agcctcaggt gtacacactg 2220
cctccatcta gggaggagat gaccaagaat caggtgtccc tgacctgtct ggtgaagggc 2280
ttctaccctt ctgatatcgc tgtggagtgg gagtctaatg gccagcccga gaacaattac 2340
aagaccaccc ctcctgtgct ggattctgac ggctccttct tcctgtactc caaactgacc 2400
gtggacaagt ctagatggca gcagggcaac gtgttctctt gttccgtgat gcacgaggct 2460

ctgcacaatc actataccca gaagtccctg tctctgtctc ctggcaagtg a 2511

<210> 13
<211> 835
<212> PRT
<213> artificial

<220>
<223> sgp130Fc_opt

<400> 13

```
Met Leu Thr Leu Gln Thr Trp Leu Val Gln Ala Leu Phe Ile Phe Leu
1               5                  10                  15

Thr Thr Glu Ser Thr Gly Glu Leu Leu Asp Pro Cys Gly Tyr Ile Ser
            20                  25                  30

Pro Glu Ser Pro Val Val Gln Leu His Ser Asn Phe Thr Ala Val Cys
        35                  40                  45

Val Leu Lys Glu Lys Cys Met Asp Tyr Phe His Val Asn Ala Asn Tyr
    50                  55                  60

Ile Val Trp Lys Thr Asn His Phe Thr Ile Pro Lys Glu Gln Tyr Thr
65                  70                  75                  80

Ile Ile Asn Arg Thr Ala Ser Ser Val Thr Phe Thr Asp Ile Ala Ser
                85                  90                  95

Leu Asn Ile Gln Leu Thr Cys Asn Ile Leu Thr Phe Gly Gln Leu Glu
            100                 105                 110

Gln Asn Tyr Tyr Gly Ile Thr Ile Ile Ser Gly Leu Pro Pro Glu Lys
        115                 120                 125

Pro Lys Asn Leu Ser Cys Ile Val Asn Glu Gly Lys Lys Met Arg Cys
    130                 135                 140

Glu Trp Asp Gly Gly Arg Glu Thr His Leu Glu Thr Asn Phe Thr Leu
145                 150                 155                 160

Lys Ser Glu Trp Ala Thr His Lys Phe Ala Asp Cys Lys Ala Lys Arg
                165                 170                 175

Asp Thr Pro Thr Ser Cys Thr Val Asp Tyr Ser Thr Val Tyr Phe Val
            180                 185                 190

Asn Ile Glu Val Trp Val Glu Ala Glu Asn Ala Leu Gly Lys Val Thr
        195                 200                 205

Ser Asp His Ile Asn Phe Asp Pro Val Tyr Lys Val Lys Pro Asn Pro
```

210 215 220

Pro His Asn Leu Ser Val Ile Asn Ser Glu Glu Leu Ser Ser Ile Leu
225 230 235 240

Lys Leu Thr Trp Thr Asn Pro Ser Ile Lys Ser Val Ile Ile Leu Lys
245 250 255

Tyr Asn Ile Gln Tyr Arg Thr Lys Asp Ala Ser Thr Trp Ser Gln Ile
260 265 270

Pro Pro Glu Asp Thr Ala Ser Thr Arg Ser Ser Phe Thr Val Gln Asp
275 280 285

Leu Lys Pro Phe Thr Glu Tyr Val Phe Arg Ile Arg Cys Met Lys Glu
290 295 300

Asp Gly Lys Gly Val Trp Ser Asp Trp Ser Glu Glu Ala Ser Gly Ile
305 310 315 320

Thr Tyr Glu Asp Arg Pro Ser Lys Ala Pro Ser Phe Trp Tyr Lys Ile
325 330 335

Asp Pro Ser His Thr Gln Gly Tyr Arg Thr Val Gln Leu Val Trp Lys
340 345 350

Thr Leu Pro Pro Phe Glu Ala Asn Gly Lys Ile Leu Asp Tyr Glu Val
355 360 365

Thr Leu Thr Arg Trp Lys Ser His Leu Gln Asn Tyr Thr Val Asn Ala
370 375 380

Thr Lys Leu Thr Val Asn Leu Thr Asn Asp Arg Tyr Leu Ala Thr Leu
385 390 395 400

Thr Val Arg Asn Leu Val Gly Lys Ser Asp Ala Ala Val Leu Thr Ile
405 410 415

Pro Ala Cys Asp Phe Gln Ala Thr His Pro Val Met Asp Leu Lys Ala
420 425 430

Phe Pro Lys Asp Asn Met Leu Trp Val Glu Trp Thr Thr Pro Arg Glu
435 440 445

Ser Val Lys Lys Tyr Ile Leu Glu Trp Cys Val Leu Ser Asp Lys Ala
450 455 460

Pro Cys Ile Thr Asp Trp Gln Gln Glu Asp Gly Thr Val His Arg Thr
465 470 475 480

Tyr Leu Arg Gly Asn Leu Ala Glu Ser Lys Cys Tyr Leu Ile Thr Val

485 490 495

Thr Pro Val Tyr Ala Asp Gly Pro Gly Ser Pro Glu Ser Ile Lys Ala
500 505 510

Tyr Leu Lys Gln Ala Pro Pro Ser Lys Gly Pro Thr Val Arg Thr Lys
515 520 525

Lys Val Gly Lys Asn Glu Ala Val Leu Glu Trp Asp Gln Leu Pro Val
530 535 540

Asp Val Gln Asn Gly Phe Ile Arg Asn Tyr Thr Ile Phe Tyr Arg Thr
545 550 555 560

Ile Ile Gly Asn Glu Thr Ala Val Asn Val Asp Ser Ser His Thr Glu
565 570 575

Tyr Thr Leu Ser Ser Leu Thr Ser Asp Thr Leu Tyr Met Val Arg Met
580 585 590

Ala Ala Tyr Thr Asp Glu Gly Gly Lys Asp Gly Pro Glu Phe Arg Ser
595 600 605

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Ala Glu
610 615 620

Gly Ala Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
625 630 635 640

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
645 650 655

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
660 665 670

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
675 680 685

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
690 695 700

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
705 710 715 720

Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
725 730 735

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
740 745 750

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val

```
                 755                 760                 765

Glu Trp Glu Ser Asn Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro
    770                 775                 780

Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val
785                 790                 795                 800

Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
                805                 810                 815

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser
            820                 825                 830

Pro Gly Lys
        835
```

Annex to the application documents - subsequently filed sequences listing

[0033]

SEQUENCE LISTING

<110> Conaris research institute AG

<120> Optimized nucleotide sequences encoding sgp130

<130> C 1028EP

<140> EP 04020455.4
<141> 2004-08-27

<160> 9

<170> PatentIn version 3.2

<210> 1
<211> 22
<212> PRT
<213> Artificial sequence

<220>
<223> secretory leader sequence of sgp130

<400> 1

Met Leu Thr Leu Gln Thr Trp Val Val Gln Ala Leu Phe Ile Phe Leu
1 5 10 15

Thr Thr Glu Ser Thr Gly
20

<210> 2
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<223> Primer

<400> 2
atgaggtgtg agtgggatgg 20

<210> 3
<211> 22
<212> DNA
<213> Artificial sequence

<220>
<223> Primer

<400> 3
accttgcact tgtactcctt gc 22

<210> 4
<211> 21
<212> DNA
<213> Artificial sequence

<220>
<223> Primer

<400> 4
gatgcctgct tgccgaatat c 21

<210> 5
<211> 22
<212> DNA
<213> Artificial sequence

<220>
<223> Primer

<400> 5
cgccaagctc ttcagcaata tc 22

<210> 6
<211> 918
<212> DNA
<213> Artificial sequence

<220>
<223> sgp130(d1-D3)_opt

<400> 6
atggaactgc tggacccgtg cggttatatc agcccggaaa gcccagttgt tcaactgcat 60

agcaatttca ccgcggtttg tgtgctgaaa gagaagtgta tggattattt ccacgtcaac 120

gcgaactaca ttgtgtggaa aaccaaccat ttcaccatcc cgaaagaaca gtataccatc 180

attaaccgta ccgcgagcag cgttaccttt accgatatcg cgagcctgaa cattcaactg 240

acctgcaaca ttctgacctt cggtcaactg gaacagaatg tttatggcat caccatcatt 300

agcggcctgc cgccggaaaa accgaaaaat ctgagctgca ttgtgaacga aggcaaaaaa 360

atgcgctgcg aatgggatgg tggtcgcgaa acccacctgg aaaccaactt caccctgaaa 420

agcgaatggg cgacccacaa atttgcggat tgcaaagcga aacgcgatac cccgacctct 480

tgcaccgttg attacagcac cgtgtacttc gtgaacattg aagtgtgggt ggaagcggaa 540

aacgccctgg gtaaagtcac cagcgatcat atcaactttg atccggtgta caaagtgaaa 600

ccgaatccgc cgcataatct gagcgtgatc aacagcgaag aactgagcag catcctgaaa 660

ctgacctgga ccaacccgag cattaagagc gttatcatcc tgaaatacaa cattcagtat 720

cgcaccaaag atgccagcac ctggagccag attccgccgg aagataccgc gagcacccgt 780

agcagcttca ccgttcagga tctgaaaccg tttaccgaat atgtgtttcg cattcgctgt 840

atgaaagaag atggtaaagg ctactggagc gattggagcg aagaagcgag cggtatcacc 900

tatgaagatc gtccgtga 918

<210> 7
<211> 918
<212> DNA
<213> Artificial sequence

<220>
<223> sgp130(D1-D3)_wt

<400> 7
atggaacttc tagatccatg tggttatatc agtcctgaat ctccagttgt acaacttcat 60

tctaatttca ctgcagtttg tgtgctaaag gaaaaatgta tggattattt tcatgtaaat 120

gctaattaca ttgtctggaa aacaaaccat tttactattc ctaaggagca atatactatc 180

ataaacagaa cagcatccag tgtcaccttt acagatatag cttcattaaa tattcagctc 240

acttgcaaca ttcttacatt cggacagctt gaacagaatg tttatggaat cacaataatt 300

tcgggcttgc ctccagaaaa acctaaaaat ttgagttgca ttgtgaacga ggggaagaaa 360

atgaggtgtg agtgggatgg tggaagggaa acacacttgg agacaaactt cactttaaaa 420

tctgaatggg caacacacaa gtttgctgat tgcaaagcaa aacgtgacac ccccacctca 480

tgcactgttg attattctac tgtgtatttt gtcaacattg aagtctgggt agaagcagag 540

aatgcccttg ggaaggttac atcagatcat atcaattttg atcctgtata taaagtgaag 600

cccaatccgc cacataattt atcagtgatc aactcagagg aactgtctag tatcttaaaa 660

ttgacatgga ccaacccaag tattaagagt gttataatac taaaatataa cattcaatat 720

aggaccaaag atgcctcaac ttggagccag attcctcctg aagacacagc atccacccga 780

tcttcattca ctgtccaaga ccttaaacct tttacagaat atgtgtttag gattcgctgt 840

atgaaggaag atggtaaggg atactggagt gactggagtg aagaagcaag tgggatcacc 900

tatgaagata gaccatga 918

<210> 8
<211> 305
<212> PRT
<213> Artificial sequence

<220>
<223> sgp130(D1-D3)_wt

<400> 8

Met Glu Leu Leu Asp Pro Cys Gly Tyr Ile Ser Pro Glu Ser Pro Val
1 5 10 15

Val Gln Leu His Ser Asn Phe Thr Ala Val Cys Val Leu Lys Glu Lys
20 25 30

Cys Met Asp Tyr Phe His Val Asn Ala Asn Tyr Ile Val Trp Lys Thr
35 40 45

Asn His Phe Thr Ile Pro Lys Glu Gln Tyr Thr Ile Ile Asn Arg Thr
50 55 60

Ala Ser Ser Val Thr Phe Thr Asp Ile Ala Ser Leu Asn Ile Gln Leu
65 70 75 80

Thr Cys Asn Ile Leu Thr Phe Gly Gln Leu Glu Gln Asn Val Tyr Gly
85 90 95

Ile Thr Ile Ile Ser Gly Leu Pro Pro Glu Lys Pro Lys Asn Leu Ser
100 105 110

Cys Ile Val Asn Glu Gly Lys Lys Met Arg Cys Glu Trp Asp Gly Gly

115 120 125

Arg Glu Thr His Leu Glu Thr Asn Phe Thr Leu Lys Ser Glu Trp Ala
130 135 140

Thr His Lys Phe Ala Asp Cys Lys Ala Lys Arg Asp Thr Pro Thr Ser
145 150 155 160

Cys Thr Val Asp Tyr Ser Thr Val Tyr Phe Val Asn Ile Glu Val Trp
165 170 175

Val Glu Ala Glu Asn Ala Leu Gly Lys Val Thr Ser Asp His Ile Asn
180 185 190

Phe Asp Pro Val Tyr Lys Val Lys Pro Asn Pro Pro His Asn Leu Ser
195 200 205

Val Ile Asn Ser Glu Glu Leu Ser Ser Ile Leu Lys Leu Thr Trp Thr
210 215 220

Asn Pro Ser Ile Lys Ser Val Ile Ile Leu Lys Tyr Asn Ile Gln Tyr
225 230 235 240

Arg Thr Lys Asp Ala Ser Thr Trp Ser Gln Ile Pro Pro Glu Asp Thr
245 250 255

Ala Ser Thr Arg Ser Ser Phe Thr Val Gln Asp Leu Lys Pro Phe Thr
260 265 270

Glu Tyr Val Phe Arg Ile Arg Cys Met Lys Glu Asp Gly Lys Gly Tyr
275 280 285

Trp Ser Asp Trp Ser Glu Glu Ala Ser Gly Ile Thr Tyr Glu Asp Arg
290 295 300

Pro
305

<210> 9
<211> 305
<212> PRT
<213> Artificial sequence

<220>
<223> sgp130(D-D3)_opt

<400> 9

Met Glu Leu Leu Asp Pro Cys Gly Tyr Ile Ser Pro Glu Ser Pro Val
1 5 10 15

Val Gln Leu His Ser Asn Phe Thr Ala Val Cys Val Leu Lys Glu Lys
20 25 30

Cys Met Asp Tyr Phe His Val Asn Ala Asn Tyr Ile Val Trp Lys Thr
35 40 45

Asn His Phe Thr Ile Pro Lys Glu Gln Tyr Thr Ile Ile Asn Arg Thr
50 55 60

Ala Ser Ser Val Thr Phe Thr Asp Ile Ala Ser Leu Asn Ile Gln Leu
65 70 75 80

Thr Cys Asn Ile Leu Thr Phe Gly Gln Leu Glu Gln Asn Val Tyr Gly
85 90 95

Ile Thr Ile Ile Ser Gly Leu Pro Pro Glu Lys Pro Lys Asn Leu Ser
100 105 110

Cys Ile Val Asn Glu Gly Lys Lys Met Arg Cys Glu Trp Asp Gly Gly
115 120 125

Arg Glu Thr His Leu Glu Thr Asn Phe Thr Leu Lys Ser Glu Trp Ala
130 135 140

Thr His Lys Phe Ala Asp Cys Lys Ala Lys Arg Asp Thr Pro Thr Ser
145 150 155 160

Cys Thr Val Asp Tyr Ser Thr Val Tyr Phe Val Asn Ile Glu Val Trp
165 170 175

Val Glu Ala Glu Asn Ala Leu Gly Lys Val Thr Ser Asp His Ile Asn
180 185 190

Phe Asp Pro Val Tyr Lys Val Lys Pro Asn Pro Pro His Asn Leu Ser
195 200 205

Val Ile Asn Ser Glu Glu Leu Ser Ser Ile Leu Lys Leu Thr Trp Thr
210 215 220

Asn Pro Ser Ile Lys Ser Val Ile Ile Leu Lys Tyr Asn Ile Gln Tyr
225 230 235 240

Arg Thr Lys Asp Ala Ser Thr Trp Ser Gln Ile Pro Pro Glu Asp Thr
245 250 255

Ala Ser Thr Arg Ser Ser Phe Thr Val Gln Asp Leu Lys Pro Phe Thr
260 265 270

Glu Tyr Val Phe Arg Ile Arg Cys Met Lys Glu Asp Gly Lys Gly Tyr
275 280 285

Trp Ser Asp Trp Ser Glu Glu Ala Ser Gly Ile Thr Tyr Glu Asp Arg
290 295 300

Pro
305

Claims

1. A nucleic acid molecule encoding sgp130 comprising (a) the nucleic acid sequence as (a_1) depicted in Figure 2 (sgp130(D1-3)_opt) or (a_2) Figure 3 (sgp130Fc_opt) or (b) a fragment or analogue thereof which maintains the codon usage pattern thereof.

2. The nucleic acid molecule of claim 1, wherein at least 80% of the codons altered in the nucleic acid sequence of Figure 2 or 3 vs. the wild type sequence are present.

3. The nucleic acid molecule of claim 1 or 2 which is a DNA molecule.

4. An expression vector containing a nucleic acid molecule of any one of claims 1 to 3.

5. A host cell containing an expression vector of claim 4.

6. The host cell of claim 5 which is a mammalian host cell.

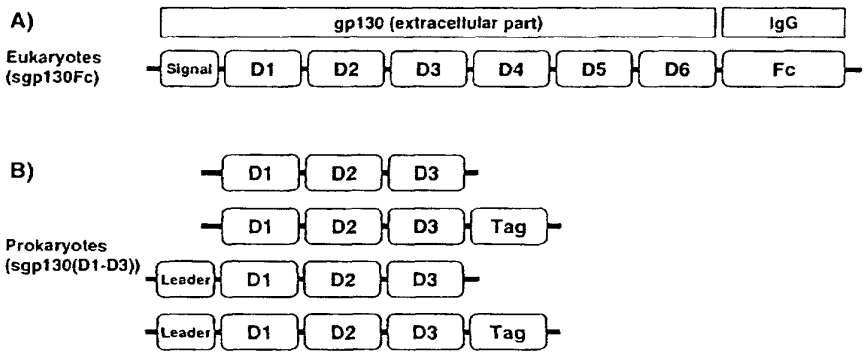
7. The host cell of claim 6 which is a CHO or HEK293 cell.

8. The host cell of claim 5 which is a prokaryotic cell.

9. The host cell of claim 8 which is a bacterial cell.

10. A method of producing a sgp130 polypeptide comprising culturing a host cell of any one of claims 5 to 9 and recovering the polypeptide from said host cell or the culture.

Figure 1


A)
gp130 (extracellular part)
IgG
Eukaryotes
(sgp130Fc)
Signal
D1
D2
D3
D4
D5
D6
Fc
B)
D1
D2
D3
D1
D2
D3
Tag
Prokaryotes
(sgp130(D1-D3))
Leader
D1
D2
D3
Leader
D1
D2
D3
Tag

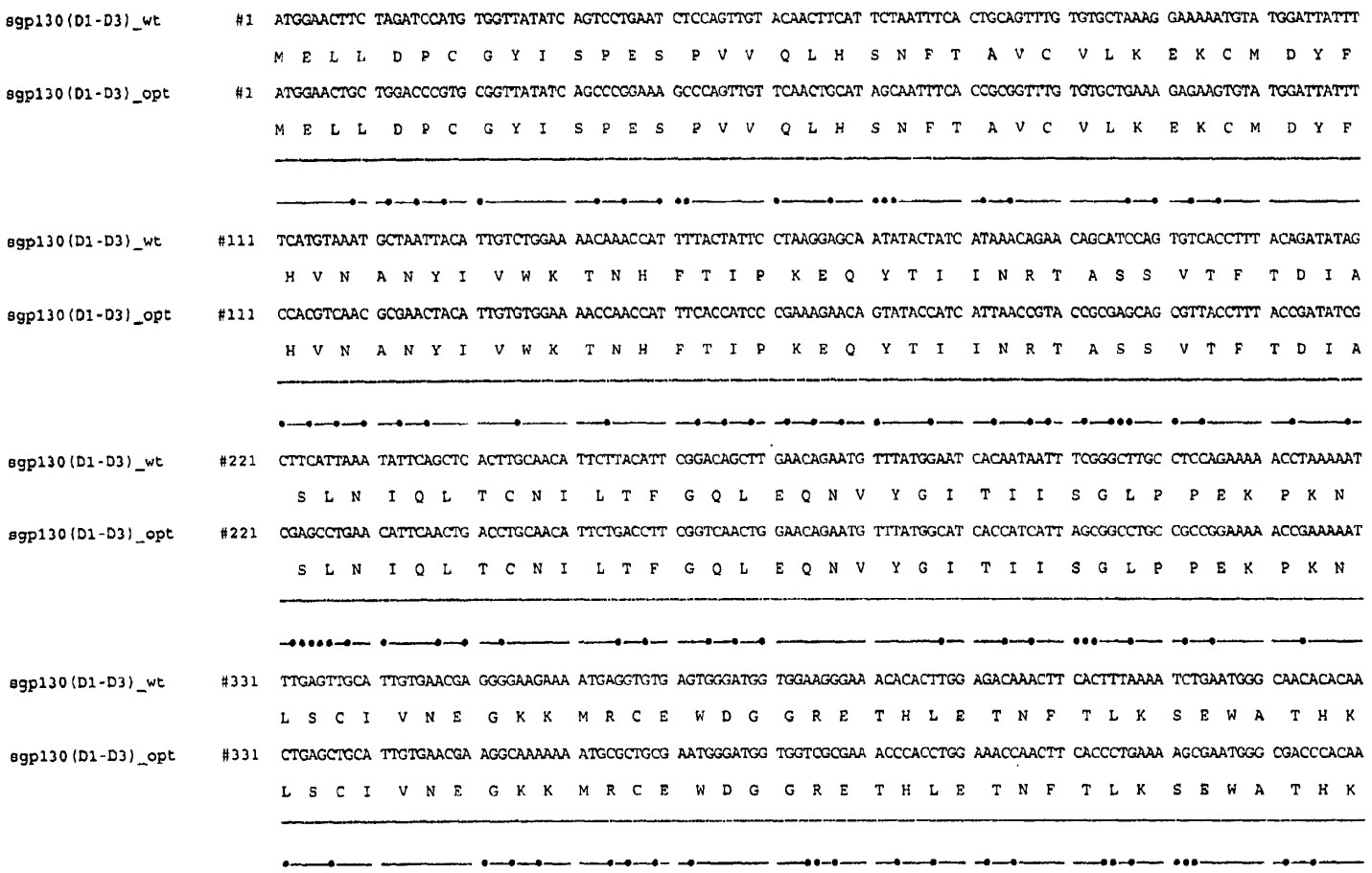

sgp130(D1-D3)_wt #1 ATGGAACTTC TAGATCCATG TGGTTATATC AGTCCTGAAT CTCCAGTTGT ACAACTTCAT TCTAATTTCA CTGCAGTTTG TGTGCTAAAG GAAAAATGTA TGGATTATTT
M E L L D P C G Y I S P E S P V V Q L H S N F T A V C V L K E K C M D Y F
sgp130(D1-D3)_opt #1 ATGGAACTGC TGGACCCGTG CGGTTATATC AGCCCGGAAA GCCCAGTTGT TCAACTGCAT AGCAATTTCA CCGCGGTTTG TGTGCTGAAA GAGAAGTGTA TGGATTATTT
M E L L D P C G Y I S P E S P V V Q L H S N F T A V C V L K E K C M D Y F
sgp130(D1-D3)_wt #111 TCATGTAAAT GCTAATTACA TTGTCTGGAA AACAAACCAT TTTACTATTC CTAAGGAGCA ATATACTATC ATAAACAGAA CAGCATCCAG TGTCACCTTT ACAGATATAG
H V N A N Y I V W K T N H F T I P K E Q Y T I I N R T A S S V T F T D I A
sgp130(D1-D3)_opt #111 CCACGTCAAC GCGAACTACA TTGTGTGGAA AACCAACCAT TTCACCATCC CGAAAGAACA GTATACCATC ATTAACCGTA CCGCGAGCAG CGTTACCTTT ACCGATATCG
H V N A N Y I V W K T N H F T I P K E Q Y T I I N R T A S S V T F T D I A
sgp130(D1-D3)_wt #221 CTTCATTAAA TATTCAGCTC ACTTGCAACA TTCTTACATT CGGACAGCTT GAACAGAATG TTTATGGAAT CACAATAATT TCGGGCTTGC CTCCAGAAAA ACCTAAAAAT
S L N I Q L T C N I L T F G Q L E Q N V Y G I T I I S G L P P E K P K N
sgp130(D1-D3)_opt #221 CGAGCCTGAA CATTCAACTG ACCTGCAACA TTCTGACCTT CGGTCAACTG GAACAGAATG TTTATGGCAT CACCATCATT AGCGGCCTGC CGCCGGAAAA ACCGAAAAAT
S L N I Q L T C N I L T F G Q L E Q N V Y G I T I I S G L P P E K P K N
sgp130(D1-D3)_wt #331 TTGAGTTGCA TTGTGAACGA GGGGAAGAAA ATGAGGTGTG AGTGGGATGG TGGAAGGGAA ACACACTTGG AGACAAACTT CACTTTAAAA TCTGAATGGG CAACACACAA
L S C I V N E G K K M R C E W D G G R E T H L E T N F T L K S E W A T H K
sgp130(D1-D3)_opt #331 CTGAGCTGCA TTGTGAACGA AGGCAAAAAA ATGCGCTGCG AATGGGATGG TGGTCGCGAA ACCCACCTGG AAACCAACTT CACCCTGAAA AGCGAATGGG CGACCCACAA
L S C I V N E G K K M R C E W D G G R E T H L E T N F T L K S E W A T H K

Fig. 2

```
gp130(D1-D3)_wt     #441  GTTTGCTGAT TGCAAAGCAA AACGTGACAC CCCCACCTCA TGCACTGTTG ATTATTCTAC TGTGTATTTT GTCAACATTG AAGTCTGGGT AGAAGCAGAG AATGCCCTTG
                          F  A  D  C  K  A  K  R  D  T  P  T  S  C  T  V  D  Y  S  T  V  Y  F  V  N  I  E  V  W  V  E  A  E  N  A  L  G

gp130(D1-D3)_opt    #441  ATTTGCGGAT TGCAAAGGCA AACGCGATAC CCCGACCTCT TGCACCGTTG ATTACAGCAC CGTGTACTTC GTCAACATTG AAGTGTGGGT CGAAGCGGAA AACGCCCTGG
                          F  A  D  C  K  A  K  R  D  T  P  T  S  C  T  V  D  Y  S  T  V  Y  F  V  N  I  E  V  W  V  E  A  E  N  A  L  G

gp130(D1-D3)_wt     #551  GGAAGGTTAC ATCAGATCAT ATCAATTTTG ATCCTGTATA TAAAGTGAAG CCCAATCCGC CACATAATTT ATCAGTGATC AACTCAGAGG AACTGTCTAG TATCTTAAAA
                          K  V  T  S  D  H  I  N  F  D  P  V  Y  K  V  K  P  N  P  P  H  N  L  S  V  I  N  S  E  E  L  S  S  I  L  K

gp130(D1-D3)_opt    #551  GTAAAGTCAC CAGCGATCAT ATCAACTTTG ATCCGGTGTA CAAAGTGAAA CCGAATCCGC CGCATAATCT GAGCGTGATC AACAGCGAAG AACTGAGCAG CATCCTGAAA
                          K  V  T  S  D  H  I  N  F  D  P  V  Y  K  V  K  P  N  P  P  H  N  L  S  V  I  N  S  E  E  L  S  S  I  L  K

gp130(D1-D3)_wt     #661  TTGACATGGA CCAACCCAAG TATTAAGAGT GTTATAATAC TAAAATATAA CATTCAATAT AGGACCAAAG ATGCCTCAAC TTGGAGCCAG ATTCCTCCTG AAGACACAGC
                          L  T  W  T  N  P  S  I  K  S  V  I  I  L  K  Y  N  I  Q  Y  R  T  K  D  A  S  T  W  S  Q  I  P  P  E  D  T  A

gp130(D1-D3)_opt    #661  CTGACCTGGA CCAACCCGAG CATTAAGAGC GTTATCATCC TGAATACAA CATTCAGTAT CGCACCAAAG ATGCCAGCAC CTGGAGCCAG ATTCCGCCGG AAGATACCGC
                          L  T  W  T  N  P  S  I  K  S  V  I  I  L  K  Y  N  I  Q  Y  R  T  K  D  A  S  T  W  S  Q  I  P  P  E  D  T  A

gp130(D1-D3)_wt     #771  ATCCACCCGA TCTTCATTCA CTGTCCAAGA CCTTAAACCT TTTACAGAAT ATGTGTTTAG GATTCGCTGT ATGAAGGAAG ATGGTAAGGG ATACTGGAGT GACTGGAGTG
                          S  T  R  S  S  F  T  V  Q  D  L  K  P  F  T  E  Y  V  F  R  I  R  C  M  K  E  D  G  K  G  Y  W  S  D  W  S  E

gp130(D1-D3)_opt    #771  GAGCACCCGT AGCAGCTTCA CCGTTCAGGA TCTGAAACCG TTTACCGAAT ATGTGTTTCG CATTCGCTGT ATGAAAGAAG ATGGTAAAGG CTACTGGAGC GATTGGAGCG
                          S  T  R  S  S  F  T  V  Q  D  L  K  P  F  T  E  Y  V  F  R  I  R  C  M  K  E  D  G  K  G  Y  W  S  D  W  S  E
```

```
sgp130(D1-D3)_wt    #881  AAGAAGCAAG TGGGATCACC TATGAAGATA GACCATGA
                          E  A  S  G  I  T  Y  E  D  R  P  .

sgp130(D1-D3)_opt   #881  AAGAAGCCAG CGGTATCACC TATGAAGATC GTCCGTGA
                          E  A  S  G  I  T  Y  E  D  R  P  .
```

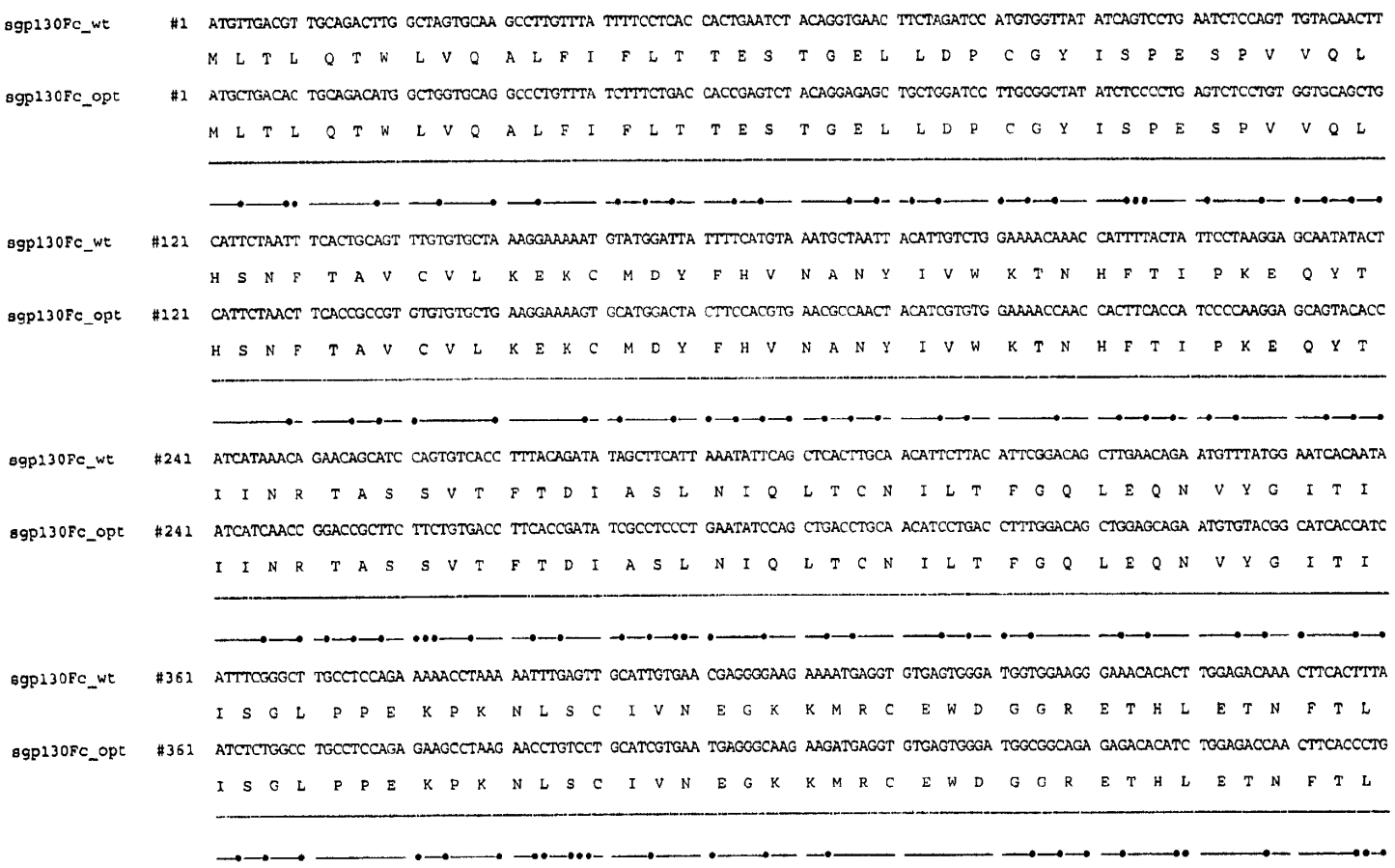

sgp130Fc_wt #1 ATGTTGACGT TGCAGACTTG GCTAGTGCAA GCCTTGTTTA TTTTCCTCAC CACTGAATCT ACAGGTGAAC TTCTAGATCC ATGTGGTTAT ATCAGTCCTG AATCTCCAGT TGTACAACTT
M L T L Q T W L V Q A L F I F L T T E S T G E L L D P C G Y I S P E S P V V Q L
sgp130Fc_opt #1 ATGCTGACAC TGCAGACATG GCTGGTGCAG GCCCTGTTTA TCTTTCTGAC CACCGAGTCT ACAGGAGAGC TGCTGGATCC TTGCGGCTAT ATCTCCCCTG AGTCTCCTGT GGTGCAGCTG
M L T L Q T W L V Q A L F I F L T T E S T G E L L D P C G Y I S P E S P V V Q L
sgp130Fc_wt #121 CATTCTAATT TCACTGCAGT TTGTGTGCTA AAGGAAAAAT GTATGGATTA TTTTCATGTA AATGCTAATT ACATTGTCTG GAAAACAAAC CATTTTACTA TTCCTAAGGA GCAATATACT
H S N F T A V C V L K E K C M D Y F H V N A N Y I V W K T N H F T I P K E Q Y T
sgp130Fc_opt #121 CATTCTAACT TCACCGCCGT GTGTGTGCTG AAGGAAAAGT GCATGGACTA CTTCCACGTG AACGCCAACT ACATCGTGTG GAAAACCAAC CACTTCACCA TCCCCAAGGA GCAGTACACC
H S N F T A V C V L K E K C M D Y F H V N A N Y I V W K T N H F T I P K E Q Y T
sgp130Fc_wt #241 ATCATAAACA GAACAGCATC CAGTGTCACC TTTACAGATA TAGCTTCATT AAATATTCAG CTCACTTGCA ACATTCTTAC ATTCGGACAG CTTGAACAGA ATGTTTATGG AATCACAATA
I I N R T A S S V T F T D I A S L N I Q L T C N I L T F G Q L E Q N V Y G I T I
sgp130Fc_opt #241 ATCATCAACC GGACCGCTTC TTCTGTGACC TTCACCGATA TCGCCTCCCT GAATATCCAG CTGACCTGCA ACATCCTGAC CTTTGGACAG CTGGAGCAGA ATGTGTACGG CATCACCATC
I I N R T A S S V T F T D I A S L N I Q L T C N I L T F G Q L E Q N V Y G I T I
sgp130Fc_wt #361 ATTTCGGGCT TGCCTCCAGA AAAACCTAAA AATTTGAGTT GCATTGTGAA CGAGGGGAAG AAAATGAGGT GTGAGTGGGA TGGTGGAAGG GAAACACACT TGGAGACAAA CTTCACTTTA
I S G L P P E K P K N L S C I V N E G K K M R C E W D G G R E T H L E T N F T L
sgp130Fc_opt #361 ATCTCTGGCC TGCCTCCAGA GAAGCCTAAG AACCTGTCCT GCATCGTGAA TGAGGGCAAG AAGATGAGGT GTGAGTGGGA TGGCGGCAGA GAGACACATC TGGAGACCAA CTTCACCCTG
I S G L P P E K P K N L S C I V N E G K K M R C E W D G G R E T H L E T N F T L

Fig. 3

```
sgp130Fc_wt    #481  AAATCTGAAT GGGCAACACA CAAGTTTGCT GATTGCAAAG CAAAACGTGA CACCCCCACC TCATGCACTG TTGATTATTC TACTGTGTAT TTTGTCAACA TTGAAGTCTG GGTAGAAGCA
                     K  S  E  W  A  T  H  K  F  A  D  C  K  A  K  R  D  T  P  T  S  C  T  V  D  Y  S  T  V  Y  F  V  N  I  E  V  W  V  E  A
sgp130Fc_opt   #481  AAGTCTGAGT GGGCTACCCA CAAGTTTGCC GACTGCAAGG CCAAGAGAGA TACCCCCTACC TCTTGCACCG TGGACTACTC CACCGTGTAC TTCGTGAACA TCGAGTGTGG GGTGGAGGCT
                     K  S  E  W  A  T  H  K  F  A  D  C  K  A  K  R  D  T  P  T  S  C  T  V  D  Y  S  T  V  Y  F  V  N  I  E  V  W  V  E  A

sgp130Fc_wt    #601  GAGAATGCCC TTGGGAAGGT TACATCAGAT CATATCAATT TTGATCCTGT ATATAAAGTG AAGCCCAATC CGCCAGATAA TTTATACAGTG ATCAACTCAG AGGAACTGTC TAGTATCTTA
                     E  N  A  L  G  K  V  T  S  D  H  I  N  F  D  P  V  Y  K  V  K  P  N  P  P  H  N  L  S  V  I  N  S  E  E  L  S  S  I  L
sgp130Fc_opt   #601  GAGAATGCTC TGGGCAAGGT GACCTCTGAC CACATCAACT TCGACCCCGT GTACAAGGTG AAGCCTAACC CTCCTCACAA CCTGTCCGTG ATCAACTCTG AGGAGCTGTC CTCTATCCTG
                     E  N  A  L  G  K  V  T  S  D  H  I  N  F  D  P  V  Y  K  V  K  P  N  P  P  H  N  L  S  V  I  N  S  E  E  L  S  S  I  L

sgp130Fc_wt    #721  AAATTGACAT GGACCAACCC AAGTATTAAG AGTGTTATAA TACTAAAATA TAACATTCAA TATAGGACCA AAGATGCCTC AACTTGGAGC CAGATTCCTC CTGAAGACAC AGGATCCACC
                     K  L  T  W  T  N  P  S  I  K  S  V  I  I  L  K  Y  N  I  Q  Y  R  T  K  D  A  S  T  W  S  Q  I  P  P  E  D  T  A  S  T
sgp130Fc_opt   #721  AAGCTGACCT GGACCAACCC TTCCATCAAG TCCGTGATCA TCCTGAAGTA CAACATCCAG TACAGGACCA AGGATGCTTC TACCTGGTCT CAGATCCCTC CTGAGGATAC CGCTTCCACC
                     K  L  T  W  T  N  P  S  I  K  S  V  I  I  L  K  Y  N  I  Q  Y  R  T  K  D  A  S  T  W  S  Q  I  P  P  E  D  T  A  S  T

sgp130Fc_wt    #841  CGATCTTCAT TCACTGTCCA AGACCTTAAA CCTTTTACAG AATATGTGTT TAGGATTCGC TGTATGAACG AAGATGGTAA GGGATACTGG AGTGACTGGA GTGAAGAAGC AAGTGGGATC
                     R  S  S  F  T  V  Q  D  L  K  P  F  T  E  Y  V  F  R  I  R  C  M  K  E  D  G  K  G  Y  W  S  D  W  S  E  E  A  S  G  I
sgp130Fc_opt   #841  AGATCCAGCT TCACAGTGCA GGACCTGAAG CCTTTTACCG AGTACGTGTT CAGGATCCGG TGCATGAAGG AGGATGGCAA GGGCTATTGG TCTGACTGGT CTGAGGAGGC TTCTGGCATC
                     R  S  S  F  T  V  Q  D  L  K  P  F  T  E  Y  V  F  R  I  R  C  M  K  E  D  G  K  G  Y  W  S  D  W  S  E  E  A  S  G  I
```

```
sgp130Fc_wt   #1441 TATTTAAGAG GGAACTTAGC AGAGAGCAAA TGCTATTTGA TAACAGTTAC TCCAGTATAT GCTGATGGAC CAGGAAGCCC TGAATCCATA AAGGCATACC TTAAACAAGC TCCACCTTCC
                    Y  L  R  G   N  L  A   E  S  K   C  Y  L  I   T  V  T   P  V  Y   A  D  G  P   G  S  P   E  S  I   K  A  Y  L   K  Q  A   P  P  S
sgp130Fc_opt  #1441 TACCTGAGAG GCAATCTGGC CGAGTCTAAG TGCTATCTGA TCACCGTGAC CCCTGTGTAT GCTGATGGAC CTGGCTCTCC TGAGTCTATC AAGGCCTACC TGAAGCAGGC TCCTCCATCT
                    Y  L  R  G   N  L  A   E  S  K   C  Y  L  I   T  V  T   P  V  Y   A  D  G  P   G  S  P   E  S  I   K  A  Y  L   K  Q  A   P  P  S

sgp130Fc_wt   #1561 AAAGGACCTA CTGTTCGGAC AAAAAAAGTA GGGAAAAACG AAGCTGTCTT AGAGTGGGAC CAACTTCCTG TTGATGTTCA GAATGGATTT ATCAGAAATT ATACTATATT TTATAGAACC
                    K  G  P  T   V  R  T   K  K  V   G  K  N  E   A  V  L   E  W  D   Q  L  P  V   D  V  Q   N  G  F   I  R  N  Y   T  I  F   Y  R  T
sgp130Fc_opt  #1561 AAGGGACCTA CCGTGAGGAC AAAGAAGGTG GGCAAGAACG AGGCTGTGCT GGAGTGGGAT CAGCTGCCTG TGGATGTGCA GAACGGCTTC ATCCGGAACT ACACCATCTT CTACCGGACC
                    K  G  P  T   V  R  T   K  K  V   G  K  N  E   A  V  L   E  W  D   Q  L  P  V   D  V  Q   N  G  F   I  R  N  Y   T  I  F   Y  R  T

sgp130Fc_wt   #1681 ATCATTGGAA ATGAAACTGC TGTGAATGTG GATTCTTCCC ACACAGAATA TACATTGTCC TCTTTGACTA GTGACACATT GTACATGGTA CGAATGGCAG CATACACAGA TGAAGGTGGG
                    I  I  G  N   E  T  A   V  N  V   D  S  S  H   T  E  Y   T  L  S   S  L  T  S   D  T  L   Y  M  V   R  M  A  A   Y  T  D   E  G  G
sgp130Fc_opt  #1681 ATCATCGGCA ATGAGACCGC CGTGAACGTG GATTCTTCCC ACACCGAGTA CACACTGTCC TCTCTGACCT CTGACACCCT GTACATGGTG AGAATGGCCG CTTATACCGA TGAGGGCGGC
                    I  I  G  N   E  T  A   V  N  V   D  S  S  H   T  E  Y   T  L  S   S  L  T  S   D  T  L   Y  M  V   R  M  A  A   Y  T  D   E  G  G

sgp130Fc_wt   #1801 AAGGATGGTC CAGAATTCAG ATCTTGTGAC AAAACTCACA CATGCCCACC GTGCCCAGCA CCTGAAGCCG AGGGCGCGCC GTCAGTCTTC CTCTTCCCCC CAAAACCCAA GGACACCCTC
                    K  D  G  P   E  F  R   S  C  D   K  T  H  T   C  P  P   C  P  A   P  E  A  E   G  A  P   S  V  F   L  F  P  P   K  P  K   D  T  L
sgp130Fc_opt  #1801 AAGGATGGAC CTGAGTTCAG ATCCTGCGAC AAGACCCACA CCTGTCCTCC TTGTCCTGCT CCTGAGGCTG AGGGCGCTCC TTCTGTGTTT CTGTTCCCCC CAAAGCCTAA GGATACCCTG
                    K  D  G  P   E  F  R   S  C  D   K  T  H  T   C  P  P   C  P  A   P  E  A  E   G  A  P   S  V  F   L  F  P  P   K  P  K   D  T  L
```

```
gp130Fc_wt      #1921  ATGATCTCCC GGACCCCTGA GGTCACATGC GTGGTGGTGG ACGTGAGCCA CGAAGACCCT GAGGTCAAGT TCAACTGGTA CGTGGACGGC GTGGAGGTGC ATAATGCCAA GACAAAGCCG
                        M  I  S  R  T  P  E  V  T  C  V  V  V  D  V  S  H  E  D  P  E  V  K  F  N  W  Y  V  D  G  V  E  V  H  N  A  K  T  K  P

gp130Fc_opt     #1921  ATGATTCTCA GAACCCCTGA GGTGACATGT GTGGTGGTGG ACGTGTCTCA TGAGGACCCC GAGGTGAAGT TCAACTGGTA CGTGGATGGC GTGGAGGTGC ACAATGCTAA GACCAAGCCT
                        M  I  S  R  T  P  E  V  T  C  V  V  V  D  V  S  H  E  D  P  E  V  K  F  N  W  Y  V  D  G  V  E  V  H  N  A  K  T  K  P

gp130Fc_wt      #2041  CGGGAGGAGC AGTACAACAG CACGTACCGT GTGGTCAGCG TCCTCACCGT CCTGCACCAG GACTGGCTGA ATGGCAAGGA GTACAAGTGC AAGGTCTCCA ACAAAGCCCT CCCAGCCCCC
                        R  E  E  Q  Y  N  S  T  Y  R  V  V  S  V  L  T  V  L  H  Q  D  W  L  N  G  K  E  Y  K  C  K  V  S  N  K  A  L  P  A  P

gp130Fc_opt     #2041  AGGGAGGAGC AGTACAACTC CACCTACAGA GTGGTGTCTG TGCTGACAGT GCTGCATCAG GATTGGCTGA ACGGCAAGGA GTACAAGTGC AAGGTGTCCA ACAAGGCTCT GCCTGCTCCT
                        R  E  E  Q  Y  N  S  T  Y  R  V  V  S  V  L  T  V  L  H  Q  D  W  L  N  G  K  E  Y  K  C  K  V  S  N  K  A  L  P  A  P

gp130Fc_wt      #2161  ATCGAGAAAA CCATCTCCAA AGCCAAAGGG CAGCCCCGAG AACCACAGGT GTACACCCTG CCCCCATCCC GGGAGGAGAT GACCAAGAAC CAGGTCAGCC TGACCTGCCT GGTCAAAGGC
                        I  E  K  T  I  S  K  A  K  G  Q  P  R  E  P  Q  V  Y  T  L  P  P  S  R  E  E  M  T  K  N  Q  V  S  L  T  C  L  V  K  G

gp130Fc_opt     #2161  ATCGAAAAGA CCATCTCCAA GGCTAAGGGA CAGCCTAGAG AGCCTCAGGT GTACACACTG CCTCCATCTA GGGAGGAGAT GACCAAGAAT CAGGTGTCCC TGACCTGTCT GGTGAAGGGC
                        I  E  K  T  I  S  K  A  K  G  Q  P  R  E  P  Q  V  Y  T  L  P  P  S  R  E  E  M  T  K  N  Q  V  S  L  T  C  L  V  K  G

gp130Fc_wt      #2281  TTCTATCCCA GCGACATTGC CGTGGAGTGG GAGAGCAATG GGCAGCCGGA GAACAACTAC AAGACCACGC CTCCCGTGCT GGACTCCGAC GGCTCCTTCT TCCTCTATAG CAAGCTCACC
                        F  Y  P  S  D  I  A  V  E  W  E  S  N  G  Q  P  E  N  N  Y  K  T  T  P  P  V  L  D  S  D  G  S  F  F  L  Y  S  K  L  T

gp130Fc_opt     #2281  TTCTACCCTT CTGATATTGC TGTGGAGTGG GAGTCTAATG GCCAGCCCGA GAACAATTAC AAGACCACCC CTCCTGTGCT GGATTCTGAC GGCTCCTTCT TCCTGTACTC CAAACTGACC
                        F  Y  P  S  D  I  A  V  E  W  E  S  N  G  Q  P  E  N  N  Y  K  T  T  P  P  V  L  D  S  D  G  S  F  F  L  Y  S  K  L  T
```

```
gp130Fc_wt   #2401  GTGGACAAGA GCAGGTGGCA GCAGGGGAAC GTCTTCTCAT GCTCCGTGAT GCATGAGGCT CTGCACAACC ACTACACGCA GAAGAGCCTC TCCCTGTCTC CGGGTAAATG A

                    V  D  K  S  R  W  Q  Q  G  N  V  F  S  C  S  V  M  H  E  A  L  H  N  H  Y  T  Q  K  S  L  S  L  S  P  G  K  .

gp130Fc_opt  #2401  GTGGACAAGT CTAGATGGCA GCAGGGCAAC GTGTTCTCTT GTTCCGTGAT GCACGAGGCT CTGCACAATC ACTATACCCA GAAGTCCCTG TCTCTGTCTC CTGGCAAGTG A

                    V  D  K  S  R  W  Q  Q  G  N  V  F  S  C  S  V  M  H  E  A  L  H  N  H  Y  T  Q  K  S  L  S  L  S  P  G  K  .
```

Figure 4

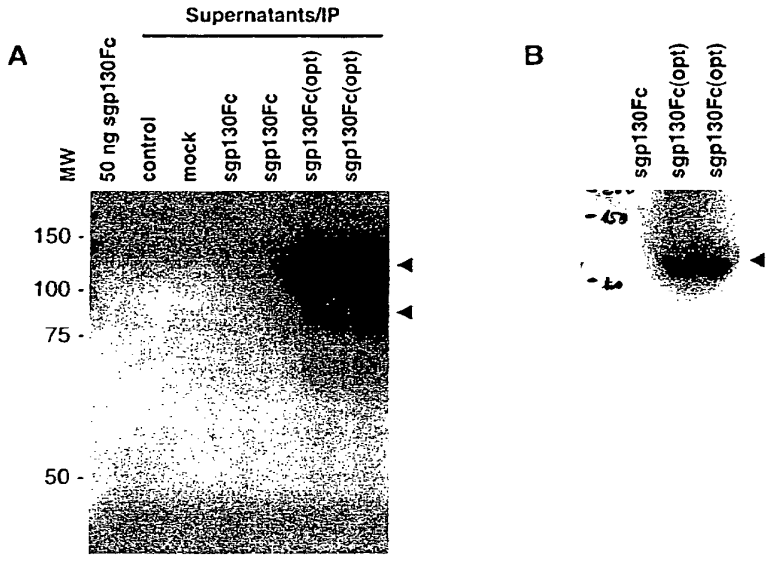
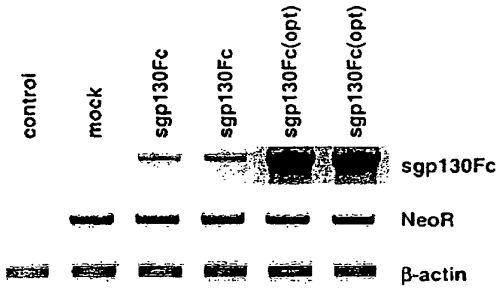


Figure 5


control
mock
sgp130Fc
sgp130Fc
sgp130Fc(opt)
sgp130Fc(opt)
sgp130Fc
NeoR
β-actin

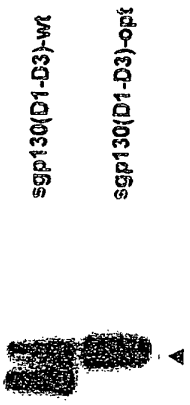


Fig.6

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 04 02 0455

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	US 2002/012962 A1 (STAHL NEIL ET AL) 31 January 2002 (2002-01-31) * paragraphs [0083], [0084]; claims; figures 4,7,8,10; examples *	1-10	C12N15/67 C07K14/715
X	WO 95/33059 A (IMMUNEX CORPORATION) 7 December 1995 (1995-12-07) * page 16; claims; examples; sequence 1 *	1-10	
X	EP 1 148 065 A (ROSE-JOHN, STEFAN) 24 October 2001 (2001-10-24) * paragraph [0033]; claims; figure 1; examples *	1-10	
E	WO 2004/113383 A (CONARIS RESEARCH INSTITUTE AG; SEEGERT, DIRK; SCHREIBER, STEFAN; ROSE-) 29 December 2004 (2004-12-29) * the whole document * & EP 1 491 554 A (CONARIS RESEARCH INSTITUTE AG) 29 December 2004 (2004-12-29)	1-10	
A	CHOW D ET AL: "IN VITRO RECONSTITUTION OF RECOGNITION AND ACTIVATION COMPLEXES BETWEEN INTERLEUKIN-6 AND GP130" BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY. EASTON, PA, US, vol. 40, no. 25, 26 June 2001 (2001-06-26), pages 7593-7603, XP001106233 ISSN: 0006-2960 * the whole document *		

-/--

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

C07K
C12N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
The Hague	8 June 2005	Madruga, J

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 04 02 0455

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	HORSTEN URSULA ET AL: "The membrane distal half of gp130 is responsible for the formation of a ternary complex with IL-6 and the IL-6 receptor" FEBS LETTERS, vol. 360, no. 1, 1995, pages 43-46, XP002331028 ISSN: 0014-5793 * the whole document *		
A	ANCEY CECILE ET AL: "A fusion protein of the gp130 and interleukin-6Ralpha ligand-binding domains acts as a potent interleukin-6 inhibitor." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 278, no. 19, 9 May 2003 (2003-05-09), pages 16968-16972, XP002330983 ISSN: 0021-9258 * the whole document *		
A	TANAKA M ET AL: "CLONING OF NOVEL SOLUBLE GP130 AND DETECTION OF ITS NEUTRALIZING AUTOANTIBODIES IN RHEUMATOID ARTHRITIS" JOURNAL OF CLINICAL INVESTIGATION, NEW YORK, NY, US, vol. 106, no. 1, July 2000 (2000-07), pages 137-144, XP000944750 ISSN: 0021-9738 * the whole document *		TECHNICAL FIELDS SEARCHED (Int.Cl.7)

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
The Hague	8 June 2005	Madruga, J

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 04 02 0455

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	KALLEN K-J: "The role of transsignalling via the agonistic soluble IL-6 receptor in human diseases" BIOCHIMICA ET BIOPHYSICA ACTA. MOLECULAR CELL RESEARCH, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 1592, no. 3, 11 November 2002 (2002-11-11), pages 323-343, XP004391610 ISSN: 0167-4889 * the whole document *		
A	FUGLSANG A: "Codon optimizer: a freeware tool for codon optimization" PROTEIN EXPRESSION AND PURIFICATION, ACADEMIC PRESS, SAN DIEGO, CA, US, vol. 31, no. 2, October 2003 (2003-10), pages 247-249, XP004462728 ISSN: 1046-5928 * the whole document *		
A	KEN-NOSUKE WADA ET AL: "CODON USAGE TABULATED FROM THE GENBANK GENETIC SEQUENCE DATA" NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 18, 25 April 1990 (1990-04-25), pages 2367-2368, XP000117389 ISSN: 0305-1048 * the whole document *		TECHNICAL FIELDS SEARCHED (Int.Cl.7)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
The Hague	8 June 2005	Madruga, J

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 04 02 0455

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

08-06-2005

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2002012962 A1	31-01-2002	AT 283365 T	15-12-2004
		AU 758970 B2	03-04-2003
		AU 6499499 A	17-04-2000
		CA 2345109 A1	06-04-2000
		CN 1357049 A	03-07-2002
		DE 69922269 D1	30-12-2004
		DK 1229047 T3	21-02-2005
		EP 1229047 A2	07-08-2002
		EP 1405915 A1	07-04-2004
		EP 1115876 A2	18-07-2001
		HK 1045847 A1	24-03-2005
		JP 2002525119 T	13-08-2002
		NO 20011513 A	25-05-2001
		NZ 510720 A	28-11-2003
		PL 348529 A1	03-06-2002
		WO 0018932 A2	06-04-2000
		US 2003143697 A1	31-07-2003
		US 2003104567 A1	05-06-2003
		US 2005074855 A1	07-04-2005
		US 2002164690 A1	07-11-2002
WO 9533059 A	07-12-1995	US 5783672 A	21-07-1998
		AU 694232 B2	16-07-1998
		AU 2601695 A	21-12-1995
		CA 2190371 A1	07-12-1995
		EP 0760857 A1	12-03-1997
		JP 10501131 T	03-02-1998
		US 5891997 A	06-04-1999
		US 6010886 A	04-01-2000
		US 5925740 A	20-07-1999
		WO 9533059 A2	07-12-1995
		US 2003109003 A1	12-06-2003
		US 6524817 B1	25-02-2003
EP 1148065 A	24-10-2001	EP 1148065 A1	24-10-2001
WO 2004113383 A	29-12-2004	EP 1491554 A1	29-12-2004
		WO 2004113383 A2	29-12-2004
EP 1491554 A	29-12-2004	EP 1491554 A1	29-12-2004
		WO 2004113383 A2	29-12-2004

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/12, C07K 14/715, A61K 38/17, C07K 16/28, C12N 5/10, A61K 39/395, G01N 33/68

A1

(11) International Publication Number: **WO 96/19574**

(43) International Publication Date: 27 June 1996 (27.06.96)

(21) International Application Number: PCT/US95/15400

(22) International Filing Date: 27 November 1995 (27.11.95)

(30) Priority Data:
08/362,304 22 December 1994 (22.12.94) US

(71) Applicant: GENETICS INSTITUTE, INC. [US/US]; 87 CambridgePark Drive, Cambridge, MA 02140 (US).

(72) Inventor: TOBIN, James, F.; 78 Clearwater Road, Newton, MA 02162 (US).

(74) Agent: BROWN, Scott, A.; Genetics Institute, Inc., 87 CambridgePark Drive, Cambridge, MA 02140 (US).

(81) Designated States: AU, CA, JP, MX, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HUMAN INTERLEUKIN-11 RECEPTOR

(57) Abstract

Polynucleotides encoding the human IL-11 receptor and fragments thereof are disclosed. IL-11 receptor proteins, methods for their production, inhibitors of binding of human IL-11 and its receptor and methods for their identification are also disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

HUMAN INTERLEUKIN-11 RECEPTOR

Field of the Invention

The present invention relates to the human interleukin-11 receptor, fragments thereof and recombinant polynucleotides and cells useful for expressing such proteins.

Background of the Invention

A variety of regulatory molecules, known as cytokines, have been identified including interleukin-11 (IL-11). The various protein forms of IL-11 and DNA encoding various forms of IL-11 activity are described in Bennett, *et al.*, USPN 5,215,895 (June 1, 1993); McCoy, *et al.*, USPN 5,270,181 (December 14, 1993); and McCoy, *et al.*, USPN 5.292,646 (March 8, 1994), all incorporated herein by reference. Thus, the term "IL-11" includes proteins having the biological activity described in these patents, whether produced by recombinant genetic engineering techniques; purified from cell sources producing the factor naturally or upon induction with other factors; or synthesized by chemical techniques; or a combination of the foregoing.

IL-11 is a pleiotropic cytokine that has been implicated in production of several biological activities including: induction of multipotential hematopoietic progenitor cell proliferation (Musashi et al. (1991) Blood, 78, 1448-1451); enhancement of megakaryocyte and platelet formation (Burstein et al. (1992) J. Cell. Physiol., 153, 305-312); stimulation of acute phase protein synthesis

(Baumann et al. (1991) J. Biol. Chem., 266, 20424-20427); inhibition of adipocyte lipoprotein lipase activity (Kawashima et al. (1991) FEBS Lett., 283, 199-202); and effects on neurotransmitter phenotype (Fann et al. (1994) Proc. Natl. Acad. Sci. USA, 91, 43-47).

IL-11 may be used in a pharmaceutical preparation or formulation to treat immune deficiencies, specifically deficiencies in hematopoietic progenitor cells, or disorders relating thereto. Treatment of the other disorders or stimulation of the immune systems of cells thereof may also employ IL-11. IL-11 may also be employed in methods for treating cancer and other disease. Such pathological states may result from disease, exposure to radiation or drugs, and include, for example, leukopenia, bacterial and viral infections, anemia, B cell or T cell deficiencies such as immune cell or hematopoietic cell deficiency following a bone marrow transplantation. IL-11 may also be used to potentiate the immune response to a variety of vaccines creating longer lasting and more effective immunity. Therapeutic treatment of cancer and other diseases with IL-11 may avoid undesirable side effects caused by treatment with presently available drugs.

Like most cytokines, IL-11 exhibits certain biological activities by interacting with an IL-11 receptor (IL-11R) on the surface of target cells. It would be desirable to identify and clone the sequence for the human receptor so that IL-11R proteins can be produced for various reasons, including production of therapeutics and screening for inhibitors of IL-11 binding to the receptor and receptor signalling.

Summary of the Invention

In accordance with the present invention, polynucleotides encoding the human interleukin-11 receptor are disclosed. In certain embodiments, the invention provides an isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence of SEQ ID NO:1 from nucleotide 803 to nucleotide 1999;

(b) a nucleotide sequence varying from the sequence of the nucleotide sequence specified in (a) as a result of degeneracy of the genetic code; and

(c) an allelic variant of the nucleotide sequence specified in (a).

Preferably, the nucleotide sequence encodes a protein having a biological activity of the human IL-11 receptor. The nucleotide sequence may be operably linked to an expression control sequence. In preferred embodiments, the polynucleotide comprises the nucleotide sequence of SEQ ID NO:1 from nucleotide 803 to nucleotide 1999 or a fragment thereof; the nucleotide sequence of SEQ ID NO:1 from nucleotide 803 to nucleotide 1828 or a fragment thereof; the nucleotide sequence of SEQ ID NO:1 from nucleotide 1904 to nucleotide 1999 or a fragment thereof; the nucleotide sequence of SEQ ID NO:1 from nucleotide 734 to nucleotide 1999 or a fragment thereof; the nucleotide sequence of SEQ ID NO:1 from nucleotide 1067 to nucleotide 1828 or a fragment thereof; or the nucleotide sequence of SEQ ID NO:1 from nucleotide 1067 to nucleotide 1999 or a fragment thereof. In other embodiments, the polynucleotide comprises a nucleotide sequence capable of

hybridizing to the nucleotides sequence of SEQ ID NO:1 under highly stringent conditions.

The invention also provides isolated polynucleotides comprising a nucleotide sequence encoding a peptide or protein comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2;

(b) the amino acid sequence of SEQ ID NO:2 from amino acids 24 to 422;

(c) the amino acid sequence of SEQ ID NO:2 from amino acids 24 to 365;

(d) the amino acid sequence of SEQ ID NO:2 from amino acids 391 to 422;

(e) the amino acid sequence of SEQ ID NO:2 from amino acids 112 to 422;

(f) the amino acid sequence of SEQ ID NO:2 from amino acids 112 to 365; and

(g) fragments of (a)-(f) having a biological activity of the human IL-11 receptor.

Host cells, preferably mammalian cells, transformed with the polynucleotides are also provided.

In other embodiments, the invention provides a process for producing a human IL-11R protein. The process comprises:

(a) growing a culture of the host cell of the present invention in a suitable culture medium; and

(b) purifying the human IL-11R protein from the culture.

Proteins produced according to these methods are also provided.

The present invention also provides for an isolated human IL-11R protein comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2;

(b) the amino acid sequence of SEQ ID NO:2 from amino acids 24 to 422;

(c) the amino acid sequence of SEQ ID NO:2 from amino acids 24 to 365;

(d) the amino acid sequence of SEQ ID NO:2 from amino acids 391 to 422;

(e) the amino acid sequence of SEQ ID NO:2 from amino acids 112 to 422;

(f) the amino acid sequence of SEQ ID NO:2 from amino acids 112 to 365; and

(g) fragments of (a)-(f) having a biological activity of the human IL-11 receptor. Preferably the protein comprises the amino acid sequence of SEQ ID NO:2; the sequence from amino acid 24 to 422 of SEQ ID NO:2; the sequence from amino acid 24 to 365 of SEQ ID NO:2; or the sequence from amino acid 391 to 422 of SEQ ID NO:2. Pharmaceutical compositions comprising a protein of the present invention and a pharmaceutically acceptable carrier are also provided.

The present invention further provides for compositions comprising an antibody which specifically reacts with a protein of the present invention.

Methods of identifying an inhibitor of IL-11 binding to the human IL-11 receptor are also provided. These methods comprise:

(a) combining a human IL-11R protein or a fragment thereof with IL-11 or a fragment thereof, said combination forming a first binding mixture;

(b) measuring the amount of binding between the protein and the IL-11 or fragment in the first binding mixture;

(c) combining a compound with the protein and the IL-11 or fragment to form a second binding mixture;

(d) measuring the amount of binding in the second binding mixture; and

(e) comparing the amount of binding in the first binding mixture with the amount of binding in the second binding mixture;

wherein the compound is capable of inhibiting IL-11 binding to the human IL-11 receptor when a decrease in the amount of binding of the second binding mixture occurs. Optionally, the first and/or second binding mixture may further comprise gp130 or a fragment thereof capable of binding to the protein of claim 11 or the IL-11 or fragment used therein. Inhibitors of IL-11R identified by these methods and pharmaceutical compositions containing them are also provided.

Methods of inhibiting binding of IL-11 to the human IL-11 receptor in a mammalian subject are also disclosed which comprise administering a therapeutically effective amount of a composition containing a human IL-11R

protein, an IL-11R inhibitor or an antibody to a human IL-11R protein. Methods of treating or preventing loss of bone mass in a mammalian subject using these compositions are also provided.

Brief Description of the Figures

Figure 1 depicts a schematic representation of the structures of the human IL-11 receptor and gp130.

Figure 2 presents data demonstrating the biological activity of a soluble form of recombinant human IL-11R protein.

Detailed Description of Preferred Embodiments

The inventors of the present application have for the first time identified and provided a polynucleotide encoding the human IL-11 receptor (human IL-11R).

SEQ ID NO:1 provides the nucleotide sequence of a cDNA encoding the human IL-11R. SEQ ID NO:2 provides the amino acid sequence of the receptor, included a putative signal sequence from amino acids 1-23. The mature human IL-11R is believed to have the sequence of amino acids 24-422 of SEQ ID NO:2.

The mature receptor has at least three distinct domains: an extracellular domain (comprising approximately amino acids 24-365 of SEQ ID NO:2), a transmembrane domain (comprising approximately amino acids 366-390 of SEQ ID NO:2) and an intracellular domain (comprising approximately amino acids 391-422 of SEQ ID NO:2). The extracellular domain is further divided into an

immunoglobulin-like domain (comprising approximately amino acids 24-111 of SEQ ID NO:2) and a type-I-cytokine domain (comprising approximately amino acids 112-365 of SEQ ID NO:2).

Soluble forms of human IL-11R protein can also be produced. Such soluble forms include without limitation proteins comprising amino acids 1-365 and 24-365 of SEQ ID NO:2. The soluble forms of the human IL-11R are further characterized by being soluble in aqueous solution, preferably at room temperature. Human IL-11R proteins comprising only the intracellular domain or a portion thereof may also be produced. Any forms of human IL-11R of less than full length are encompassed within the present invention and are referred to herein collectively as "human IL-11R" or "human IL-11R proteins." Human IL-11R proteins of less than full length may be produced by expressing a corresponding fragment of the polynucleotide encoding the full-length human IL-11R protein (SEQ ID NO:1). These corresponding polynucleotide fragments are also part of the present invention. Modified polynucleotides as described above may be made by standard molecular biology techniques, including construction of appropriate desired deletion mutants, site-directed mutagenesis methods or by the polymerase chain reaction using appropriate oligonucleotide primers.

Based upon similarity to the structure of the IL-6 receptor, it is predicted that IL-11R proteins containing only the type-I cytokine domain of the extracellular region of the full length receptor will be capable of binding IL-11 and inducing receptor signalling. As a result, IL-11R proteins comprising amino acids 112 to 365 of SEQ ID NO:2, IL-11R proteins comprising amino

acids 112 to 390 of SEQ ID NO:2, and IL-11R proteins comprising amino acids 112 to 422 of SEQ ID NO:2 are provided by the present invention. Polynucleotides encoding such proteins (such as for example a polynucleotide comprising the nucleotides sequence of SEQ ID NO:1 from nucleotide 1067 to nucleotide 1828, a polynucleotide comprising the nucleotides sequence of SEQ ID NO:1 from nucleotide 1067 to nucleotide 1906, and a polynucleotide comprising the nucleotides sequence of SEQ ID NO:1 from nucleotide 1067 to nucleotide 1999, respectively) are also provided by the invention.

For the purposes of the present invention, a protein has "a biological activity of the human IL-11 receptor" if it possess one or more of the following characteristics: (1) the ability to bind IL-11 or a fragment thereof (preferably a biologically active fragment thereof); (2) the ability to bind to cytosolic proteins or molecules involved in the signalling pathway invoked by IL-11 binding to human IL-11R; (3) the ability to produce a signal characteristic of the binding of IL-11 to human IL-11R (where the protein in question either contains a portion able to bind IL-11 or where the protein in question would produce such signal if joined to another protein which is able to bind IL-11); (4) the ability to bind to gp130 or a fragment thereof (either in the presence or absence of IL-11); (5) the ability to induce tyrosine phosphorylation of gp130; (6) the ability to induce tyrosine phosphorylation of JAK kinases; or (7) the ability to induce tyrosine phosphorylation of the STAT family of DNA binding proteins (Zhong et al. (1994) Science 264, 95-98). Preferably, the biological activity possessed by the protein is the ability to bind IL-11 or a fragment

hereof, more prefereably with a K_D of about 0.1 to about 100 nM, most preferably with a K_D of about 1 to about 10 nM.

Human IL-11R or active fragments thereof (human IL-11R proteins) may be fused to carrier molecules such as immunoglobulins. For example, soluble forms of the human IL-11R may be fused through "linker" sequences to the Fc portion of an immunoglobulin. Other fusions proteins, such as those with GST, Lex-A or MBP, may also be used.

The invention also encompasses allelic variations of the nucleotide sequence as set forth in SEQ ID NO:1, that is, naturally-occurring alternative forms of the isolated polynucleotide of SEQ ID NO: 1 which also encode human IL-11R proteins, preferably those proteins having a biological activity of human IL-11R. Also included in the invention are isolated polynucleotides which hybridize to the nucleotide sequence set forth in SEQ ID NO:1 under highly stringent conditions (for example, 0.1xSSC at 65°C). Isolated polynucleotides which encode human IL-11R proteins but which differ from the nucleotide sequence set forth in SEQ ID NO:1 by virtue of the degeneracy of the genetic code are also encompassed by the present invention. Variations in the nucleotide sequence as set forth in SEQ ID NO:1 which are caused by point mutations or by induced modifications are also included in the invention.

The isolated polynucleotides of the invention may be operably linked to an expression control sequence such as the pMT2 or pED expression vectors disclosed in Kaufman et al., Nucleic Acids Res. 19, 4485-4490 (1991), in order to produce the human IL-11R protein recombinantly. Many suitable expression control sequences are known in the art. General methods of expressing

recombinant proteins are also known and are exemplified in R. Kaufman, Methods in Enzymology 185, 537-566 (1990). As defined herein "operably linked" means enzymatically or chemically ligated to form a covalent bond between the isolated polynucleotide of the invention and the expression control sequence, in such a way that the human IL-11R protein is expressed by a host cell which has been transformed (transfected) with the ligated polynucleotide/expression control sequence.

A number of types of cells may act as suitable host cells for expression of the human IL-11R protein. Any cell type capable of expressing functional human IL-11R protein may be used. Suitable mammalian host cells include, for example, monkey COS cells, Chinese Hamster Ovary (CHO) cells, human kidney 293 cells, human epidermal A431 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other transformed primate cell lines, normal diploid cells, cell strains derived from in vitro culture of primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, HaK, Rat2, BaF3, 32D, FDCP-1, PC12, M1x or C2C12 cells.

The human IL-11R protein may also be produced by operably linking the isolated polynucleotide of the invention to suitable control sequences in one or more insect expression vectors, and employing an insect expression system. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, e.g., Invitrogen, San Diego, California, U.S.A. (the MaxBac® kit), and such methods are well known in the art, as described in Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987), incorporated herein by reference. Soluble forms of

the human IL-11R protein may also be produced in insect cells using appropriate isolated polynucleotides as described above.

Alternatively, the human IL-11R protein may be produced in lower eukaryotes such as yeast or in prokaryotes such as bacteria. Suitable yeast strains include Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces strains, *Candida,* or any yeast strain capable of expressing heterologous proteins. Suitable bacterial strains include *Escherichia coli, Bacillus subtilis, Salmonella typhimurium,* or any bacterial strain capable of expressing heterologous proteins.

Expression in bacteria may result in formation of inclusion bodies incorporating the recombinant protein. Thus, refolding of the recombinant protein may be required in order to produce active or more active material. Several methods for obtaining correctly folded heterologous proteins from bacterial inclusion bodies are known in the art. These methods generally involve solubilizing the protein from the inclusion bodies, then denaturing the protein completely using a chaotropic agent. When cysteine residues are present in the primary amino acid sequence of the protein, it is often necessary to accomplish the refolding in an environment which allows correct formation of disulfide bonds (a redox system). General methods of refolding are disclosed in Kohno, Meth. Enzym., 185:187-195 (1990). EP 0433225 and copending application USSN 08/163,877 describe other appropriate methods.

The human IL-11R protein of the invention may also be expressed as a product of transgenic animals, e.g., as a component of the milk of transgenic

cows, goats, pigs, or sheep which are characterized by somatic or germ cells containing a polynucleotide sequence encoding the human IL-11R protein.

The human IL-11R protein of the invention may be prepared by growing a culture transformed host cells under culture conditions necessary to express the desired protein. The resulting expressed protein may then be purified from the culture medium or cell extracts. Soluble forms of the human IL-11R protein of the invention can be purified from conditioned media. Membrane-bound forms of human IL-11R protein of the invention can be purified by preparing a total membrane fraction from the expressing cell and extracting the membranes with a non-ionic detergent such as Triton X-100.

The human IL-11R protein can be purified using methods known to those skilled in the art. For example, the human IL-11R protein of the invention can be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) or polyetheyleneimine (PEI) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred (e.g., S-Sepharose® columns). The purification of the human IL-11R protein from culture supernatant may also include one or more column

steps over such affinity resins as concanavalin A-agarose, heparin-toyopearl® or Cibacrom blue 3GA Sepharose®; or by hydrophobic interaction chromatography using such resins as phenyl ether, butyl ether, or propyl ether; or by immunoaffinity chromatography. Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify the human IL-11R protein. Affinity columns including IL-11 or fragments thereof or including antibodies to the IL-11R protein can also be used in purification in accordance with known methods. Some or all of the foregoing purification steps, in various combinations or with other known methods, can also be employed to provide a substantially purified isolated recombinant protein. Preferably, the isolated human IL-11R protein is purified so that it is substantially free of other mammalian proteins.

Human IL-11R proteins of the invention may also be used to screen for agents which are capable of binding to human IL-11R or interfere with the binding of IL-11 to the human IL-11R (either the extracellular or intracellular domains) and thus may act as inhibitors of normal binding and cytokine action (IL-11R inhibitors). Binding assays using a desired binding protein, immobilized or not, are well known in the art and may be used for this purpose using the human IL-11R protein of the invention. Purified cell based or protein based (cell free) screening assays may be used to identify such agents. For example, human IL-11R protein may be immobilized in purified form on a carrier and binding to purified human IL-11R protein may be measured in the presence and in the absence of potential inhibiting agents. A suitable binding

assay may alternatively employ a soluble form of human IL-11R of the invention.

In such a screening assay, a first binding mixture is formed by combining IL-11 or a fragment thereof and human IL-11R protein, and the amount of binding in the first binding mixture (B_o) is measured. A second binding mixture is also formed by combining IL-11 or a fragment thereof, human IL-11R protein, and the compound or agent to be screened, and the amount of binding in the second binding mixture (B) is measured. The amounts of binding in the first and second binding mixtures are compared, for example, by performing a calculation of the ratio B/B_o. A compound or agent is considered to be capable of inhibiting binding if a decrease in binding in the second binding mixture as compared to the first binding mixture is observed. Optionally, gp130 can be added to one or both of the binding mixtures. The formulation and optimization of binding mixtures is within the level of skill in the art, such binding mixtures may also contain buffers and salts necessary to enhance or to optimize binding, and additional control assays may be included in the screening assay of the invention.

Compounds found to reduce the binding activity of human IL-11R protein to IL-11 or its fragment to any degree, preferably by at least about 10%, more preferably greater than about 50% or more, may thus be identified and then secondarily screened in other binding assays and in vivo assays. By these means compounds having inhibitory activity for IL-11R binding which may be suitable as therapeutic agents may be identified.

Human IL-11R proteins, and polynucleotides encoding them, may also be used as diagnostic agents for detecting the expression or presence of IL-11R, IL-11 or cells expressing IL-11R or IL-11. The proteins or polynucleotides may be employed for such purpose in standard procedures for diagnostics assays using these types of materials. Suitable methods are well known to those skilled in the art.

Human IL-11R acts as a mediator of the known biological activities of IL-11. As a result, isolated human IL-11R protein and IL-11R inhibitors may be useful in treatment or modulation of various medical conditions in which IL-11 is implicated or which are effected by the activity (or lack thereof) of IL-11 (collectively "IL-11-related conditions"). IL-11-related conditions include without limitation immune deficiencies, specifically deficiencies in hematopoietic progenitor cells, or disorders relating thereto, cancer and other disease. Such pathological states may result from disease, exposure to radiation or drugs, and include, for example, leukopenia, bacterial and viral infections, anemia, B cell or T cell deficiencies such as immune cell or hematopoietic cell deficiency following a bone marrow transplantation.

It is also believed that IL-11 and IL-11R may play a role in the regulation of bone maturation and repair (Girasole et al. (1994) J. Clin. Invest. 93, 1516-1524; Passeri et al. (1992) J. Bone Miner. Res.. 7(S1), S110 Abst.; Passeri et al. (1993) J. Bone Miner. Res., 8(S1), S162 Abst.). As a result, human IL-11R protein and IL-11R inhibitors may be useful in treatment of bone loss (including that associated with osteoporosis, post-menopausal osteoporosis,

senile osteoporosis, idiopathic osteoporosis, Pagets disease, multipe myeloma, and hypogonadal conditions).

Human IL-11R protein and IL-11R inhibitors, purified from cells or recombinantly produced, may be used as a pharmaceutical composition when combined with a pharmaceutically acceptable carrier. Such a composition may contain, in addition to human IL-11R or ligand and carrier, diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier will depend on the route of administration.

The pharmaceutical composition of the invention may also contain cytokines, lymphokines, or other hematopoietic factors such as M-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-15, G-CSF, stem cell factor, and erythropoietin. The pharmaceutical composition may contain thrombolytic or anti-thrombotic factors such as plasminogen activator and Factor VIII. The pharmaceutical composition may further contain other anti-inflammatory agents. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with isolated human IL-11R protein or IL-11R inhibitor, or to minimize side effects caused by the isolated human IL-11R or IL-11R inhibitor. Conversely, isolated human IL-11R or IL-11R inhibitor may be included in formulations of the particular cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-

inflammatory agent to minimize side effects of the cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent.

The pharmaceutical composition of the invention may be in the form of a liposome in which isolated human IL-11R protein or IL-11R inhibitor is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers which in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Patent No. 4,235,871; U.S. Patent No. 4,501,728; U.S. Patent No. 4,837,028; and U.S. Patent No. 4,737,323, all of which are incorporated herein by reference.

As used herein, the term "therapeutically effective amount" means the total amount of each active component of the pharmaceutical composition or method that is sufficient to show a meaningful patient benefit, e.g., amelioration of symptoms of, healing of, or increase in rate of healing of such conditions. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

In practicing the method of treatment or use of the present invention, a therapeutically effective amount of isolated human IL-11R protein or IL-11R

inhibitor is administered to a mammal. Isolated human IL-11R protein or IL-11R inhibitor may be administered in accordance with the method of the invention either alone or in combination with other therapies such as treatments employing cytokines, lymphokines or other hematopoietic factors. When co-administered with one or more cytokines, lymphokines or other hematopoietic factors, human IL-11R protein or IL-11R inhibitor may be administered either simultaneously with the cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors, or sequentially. If administered sequentially, the attending physician will decide on the appropriate sequence of administering human IL-11R protein or IL-11R inhibitor in combination with cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors.

Administration of human IL-11R protein or IL-11R inhibitor used in the pharmaceutical composition or to practice the method of the present invention can be carried out in a variety of conventional ways, such as oral ingestion, inhalation, or cutaneous, subcutaneous, or intravenous injection. Intravenous administration to the patient is preferred.

When a therapeutically effective amount of human IL-11R protein or IL-11R inhibitor is administered orally, human IL-11R protein or IL-11R inhibitor will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% human IL-11R protein or IL-11R inhibitor, and preferably from about 25 to 90% human IL-11R

protein or IL-11R inhibitor. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of human IL-11R protein or IL-11R inhibitor, and preferably from about 1 to 50% human IL-11R protein or IL-11R inhibitor.

When a therapeutically effective amount of human IL-11R protein or IL-11R inhibitor is administered by intravenous, cutaneous or subcutaneous injection, human IL-11R protein or IL-11R inhibitor will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to human IL-11R protein or IL-11R inhibitor an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additive known to those of skill in the art.

The amount of human IL-11R protein or IL-11R inhibitor in the pharmaceutical composition of the present invention will depend upon the nature

and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of human IL-11R protein or IL-11R inhibitor with which to treat each individual patient. Initially, the attending physician will administer low doses of human IL-11R protein or IL-11R inhibitor and observe the patient's response. Larger doses of human IL-11R protein or IL-11R inhibitor may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not generally increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 0.1 μg to about 100 mg of human IL-11R protein or IL-11R inhibitor per kg body weight.

The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. It is contemplated that the duration of each application of the human IL-11R protein or IL-11R inhibitor will be in the range of 12 to 24 hours of continuous intravenous administration. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

Human IL-11R proteins of the invention may also be used to immunize animals to obtain polyclonal and monoclonal antibodies which specifically react with the human IL-11R protein and which may inhibit binding of IL-11 or fragments thereof to the receptor. Such antibodies may be obtained using the entire human IL-11R as an immunogen, or by using fragments of human IL-

11R, such as the soluble mature human IL-11R. Smaller fragments of the human IL-11R may also be used to immunize animals. The peptide immunogens additionally may contain a cysteine residue at the carboxyl terminus, and are conjugated to a hapten such as keyhole limpet hemocyanin (KLH). Additional peptide immunogens may be generated by replacing tyrosine residues with sulfated tyrosine residues. Methods for synthesizing such peptides are known in the art, for example, as in R.P. Merrifield, J.Amer.Chem.Soc. 85, 2149-2154 (1963); J.L. Krstenansky, et al., FEBS Lett. 211, 10 (1987).

Neutralizing or non-neutralizing antibodies (preferably monoclonal antibodies) binding to human IL-11R protein may also be useful therapeutics for certain tumors and also in the treatment of conditions described above. These neutralizing monoclonal antibodies may be capable of blocking IL-11 binding to the human IL-11R.

Example 1

Isolation of Human IL-11R cDNA

Generation of DNA Probes:

DNA probes derived from the murine Etl-2 sequence (SEQ ID NO:3) were obtained by PCR from murine placenta cDNA. The amino terminal probe corresponds to base pairs 418-570 and the carboxy terminal probe to base pairs 847- 1038 of the murine Etl-2 sequence. The DNA probes were gel purified and radiolabeled using α32P-dATP and α32P-dCTP.

cDNA Library Screening:

cDNA was generated from activated human PBMC using the Superscript Choice System and cloned into the EcoR1 site of ZAP II (Stratagene). The resulting phage were used to infect E. coli strain BB4. One million phage were plated on 150 mm NZCYM plates at a density of 15000 pfu/plate. Plaques were transferred to duplicate Duralose nitrocellulose filters (Stratagene). Following alkali denaturation and heat fixation the filters were pre-hybridized in 5X SSC, 5X Denhardts, 0.1% SDS, and 50 μg/ml yeast tRNA for 2 hours at 65°C. One set of filters was hybridized with the amino-terminal probe and the other set with the carboxy-terminal probe (5 x 10^5 cpm/ml) for 48 hrs at 55°C in pre-hybridization buffer. The filters were washed with 4X SSC, 0.1% SDS once at 25°C and twice at 55°C. Plaques that hybridized to both probes were identified by autoradiography.

Of the one million plaques screened two plaques hybridized to both of the probes. These plaques were picked and the phage eluted into SM media containing chloroform. The resulting phage were used to reinfect E. coli strain BB4 and plated on NZCYM plates at a density of 100-300 pfu/plate for a secondary screen.

Following the secondary screen plasmid DNA was isolated from the ZAPII plaques by excision using helper phage (Stratagene). The DNA sequence of the inserts was determined on an Applied Biosystems DNA sequencer.

Clone phIL11R14-2 containing the polynucleotide having the sequence of SEQ ID NO:1 was deposited with ATCC at accession number _____ on December 22, 1994.

Example 2

Expression of Soluble Human IL-11R Protein and Assay of Activity

A soluble form of human IL-11R protein was expressed in mammalian cells. The expresssed recombinant protein was capable of transducing a signal in BAF130-9 cells.

A portion of the full length human IL-11R sequence (nucleotides 734-1828 of SEQ ID NO:1 encoding amino acids 1-365 of SEQ ID NO:2) corresponding to a soluble form was cloned into the mammalian expression vector pED and used to transfect COSM6 cells. 40 hours after transfection conditioned media was removed, concentrated 5 fold and used in proliferation assays with the murine cell line BAF130-9 (Hibi, M. et al. (1990) Cell 63, 1149-57), a derivative of the BAFB03 cell line expressing the human gp130 signal transducer. BAF130-9 cells do not proliferate in response to IL-11 or IL-6 alone, but do proliferate in response to a combination of IL-6 and soluble IL-6R (Hibi et al., supra). BAF130-9 cells ($1x10^4$ cell in 0.1ml) were cultured in RPMI 1640 medium/10% FCS with increasing concentrations of recombinant human IL-11 in the absence or presence of 10 μl of conditioned media from mock transfected cells or cells transfected with the soluble human IL-11R sequence. After forty hours the cells were pulse-labeled with ^{3}H-thymidine (0.5

μCi/well) for eight hours and incorporated nucleotide was determined. As shown in Figure 2, BAF130-9 cells do not proliferate in response to IL-11 or soluble IL-11R alone, but do proliferate in the presence of both IL-11 and soluble IL-11R.

Other human IL-11R proteins can be tested in this model to determine whether they exhibit a "biological activity" of human IL-11R as defined herein.

Example 3

Other Systems for Determination Biological Activity of Human IL-11R Protein

Other systems can be used to determine whether a specific human IL-11R protein exhibits a "biological activity" of human IL-11R as defined herein. The following are examples of such systems.

Assays for IL-11 Binding

The ability of a human IL-11R protein to bind IL-11 or a fragment thereof can be determine by any sutiable assays which can detect such binding. Some suitable examples follow.

Binding of IL-11 to the extracellular region of the human IL-11R protein will specifically cause a rapid induction of phosphotyrosine on the receptor protein. Assays for ligand binding activity as measured by induction of phosphorylation are described below.

Alternatively, a human IL-11R protein (such as, for example, a soluble form of the extracellular domain) is produced and used to detect IL-11 binding.

For example, a DNA construct is prepared in which the extracellular domain (truncated prior, preferably immediately prior, to the predicted transmembrane domain) is ligated in frame to a cDNA encoding the hinge C_H2 and C_H3 domains of a human immunoglobulin (Ig) $\gamma 1$. This construct is generated in an appropriate expression vector for COS cells, such as pEDΔC or pMT2. The plasmid is transiently transfected into COS cells. The secreted IL-11R-Ig fusion protein is collected in the conditioned medium and purified by protein A chromatography.

The purified IL-11R-Ig fusion protein is used to demonstrate IL-11 binding in a number of applications. IL-11 can be coated onto the surface of an enzyme-linked immunosorbent assay (ELISA) plate, and then additional binding sites blocked with bovine serum albumin or casein using standard ELISA buffers. The IL-11R-Ig fusion protein is then bound to the solid-phase IL-11, and binding is detected with a secondary goat anti-human Ig conjugated to horseradish peroxidase. The activity of specifically bound enzyme can be measured with a colorimetric substrate, such as tetramethyl benzidine and absorbance readings.

IL-11 may also be expressed on the surface of cells, for example by providing a transmembrane domain or glucosyl phosphatidyl inositol (GPI) linkage. Cells expressing the membrane bound IL-11 can be identified using the IL-11R-Ig fusion protein. The soluble IL-11R-Ig fusion is bound to the surface of these cells and detected with goat anti-human Ig conjugated to a fluorochrome, such as fluorescein isothiocyanate and flow cytometry.

Interaction Trap

A yeast genetic selection method, the "interaction trap" [Gyuris et al, Cell 75:791-803, 1993], can be used to determine whether a human IL-11R protein has a biological activity of human IL-11R as defined herein. In this system, the expression of reporter genes from both LexAop-Leu2 and LexAop-LacZ relies on the interaction between the bait protein, for example in this case a species which interacts with huamn IL-11R, and the prey, for example in this case the human IL-11R protein. Thus, one can measure the strength of the interaction by the level of Leu2 or LacZ expression. The most simple method is to measure the activity of the LacZ encoded protein, β-galactosidase. This activity can be judged by the degree of blueness on the X-Gal containing medium or filter. For the quantitative measurement of β-galactosidase activity, standard assays can be found in "Methods in Yeast Genetics" Cold Spring Harbor, New York, 1990 (by Rose, M.D., Winston, F., and Hieter, P.).

In such methods, if one wishes to determine whether the human IL-11R protein interacts with a particular species (such as, for example, a cystoslic protein which binds to the intracellular domain of the human IL-11R *in vivo*), that species can be used as the "bait" in the interaction trap with the human IL-11R protein to be tested serving as the "prey", or *vice versa*.

CAT Induction System

Transcription of acute phase plasma protein genes, such as the rat β-fibrinogen gene, is activated by IL-11 in a variety of cell lines. In one

exemplary system, COSM6 cells are cotransfected with plasmids encoding the human IL-11R protein (such as the full length human IL-11R or a soluble form thereof), the human gp130 signal transducer and a reporter gene containing the 350 base pair promoter region of the rat b-fibrinogen gene fused to a reporter gene, CAT (Baumann et al. (1991) J. Biol. Chem. 266, 20424-27). The cells are stimulated with increasing concentrations of recombinant human IL-11 and transcription of the reporter gene is monitored by assaying for the presence of CAT activity.

Phosphorylation of gp130

Activity may also be determined by examining the ability of IL-11 to induce tyrosine phosphorylation of gp130 in cells transfected with a sequence encoding the human IL-11R protein (such as the full length human IL-11R or a soluble form thereof) (Luttcken et al. (1994) Science 263, 89-92).

Phosphorylation of STATs

Activity may also be determined by examining the ability of IL-11 to induce tyrosine phosphorylation of STATs (signal transducers and activators of transcription, a family of DNA binding proteins) in cells transfected with a sequence encoding the human IL-11R protein (such as the full length human IL-11R or a soluble form thereof) (Zhong et al. (1994) Science 264, 95-98).

Phosphorylation of JAK Kinases

Activity may also be determined by examining the ability of IL-11 to induce tyrosine phosphorylation of JAK kinases in cells transfected with a sequence encoding the human IL-11R protein (such as the full length human IL-11R or a soluble form thereof) (Yin et al. (1993) J. Immunol. 151: 2555-61).

All patent and literature references cited herein are incorporated by reference as if fully set forth.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Tobin, James

(ii) TITLE OF INVENTION: HUMAN INTERLUEKIN-11 RECEPTOR

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genetics Institute, Inc.
(B) STREET: 87 CambridgePark Drive
(C) CITY: Cambridge
(D) STATE: MA
(E) COUNTRY: USA
(F) ZIP: 02140

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Brown, Scott A.
(B) REGISTRATION NUMBER: 32,724
(C) REFERENCE/DOCKET NUMBER: GI5252

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617) 498-8224
(B) TELEFAX: (617) 876-5851

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2456 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 734..1999

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCGCCCACCC CCAGCCTCTG GCAGCAGCCA GGGCATCTGG ATCTGCTTAA CTACACAGCC 60

CCAGCCTGCA CCCTAGCCCC ATCCAGCTTC ACAAACTGGA GACCAACGAA GTGTCAAGAG 120

CCAGGCCCAG CTGAGTGGCC CAAGTAGCCA GACCAAGGAG CCAGGTTCAG GCGAGAAGCC 180

TGGCAGCCAG GGCAGGGGTG GGCCTCAGGG TGGGAGTGCA GGATGGGCTC AGATCCATGA 240

TGACACCCTT CCCCCAGGGT GATAAGGTCT GCCTAGGTTA ATCAGAGGCA GTGATAAGCC 300

CTGGACCAGG TGGGGGTAAA TACCAGAATT CCCAACAGCT GGACTGGAGG GGTTAATGGG 360

AGTGGCTGAG CTGGTGCCAG TGCTTGGTGC CAGGGGTGGG CGCCAAGGGC AGTGGAGGGG 420

GAGTTGCTGG CACAGTCTGT TGCCTCCGGC TTTGTTCTTG GGCCCTAAGC CCAGGACTGA 480

GATGGAGGGT GTGAGGGGGT GTGTGTGTCC GTGTGTGTGT GTGTGTGTGT GTGCGCGCGC 540

ACGCACATGC AAAGCACTGG GTATACAGTG GGAAAGGGGA CCTCAGGTCA GTTCCCGCAG 600

TGATTTCTAA CAGCCTTACC CCACTTGGTG CATCAATTTT TCTCCTAGGA AGCCTCAGTT 660

TTGGAGAGGA AGAGCCAGGC TTTAGCCTCC CATCTCAGGG GTCGGGGATT TTTGACTCTA 720

```
CCTTTCCCCA CAG ATG AGC AGC AGC TGC TCA GGG CTG AGC AGG GTC CTG      769
               Met Ser Ser Ser Cys Ser Gly Leu Ser Arg Val Leu
                 1               5                  10

GTG GCC GTG GCT ACA GCC CTG GTG TCT GCC TCC TCC CCC TGC CCC CAG    817
Val Ala Val Ala Thr Ala Leu Val Ser Ala Ser Ser Pro Cys Pro Gln
            15                  20                  25

GCC TGG GGC CCC CCA GGG GTC CAG TAT GGG CAG CCA GGC AGG TCC GTG    865
Ala Trp Gly Pro Pro Gly Val Gln Tyr Gly Gln Pro Gly Arg Ser Val
        30                  35                  40

AAG CTG TGT TGT CCT GGA GTG ACT GCC GGG GAC CCA GTG TCC TGG TTT    913
Lys Leu Cys Cys Pro Gly Val Thr Ala Gly Asp Pro Val Ser Trp Phe
    45                  50                  55                  60

CGG GAT GGG GAG CCA AAG CTG CTC CAG GGA CCT GAC TCT GGG CTA GGG    961
Arg Asp Gly Glu Pro Lys Leu Leu Gln Gly Pro Asp Ser Gly Leu Gly
                65                  70                  75

CAT GAA CTG GTC CTG GCC CAG GCA GAC AGC ACT GAT GAG GGC ACC TAC   1009
His Glu Leu Val Leu Ala Gln Ala Asp Ser Thr Asp Glu Gly Thr Tyr
            80                  85                  90

ATC TGC CAG ACC CTG GAT GGT GCA CTT GGG GGC ACA GTG ACC CTG CAG   1057
Ile Cys Gln Thr Leu Asp Gly Ala Leu Gly Gly Thr Val Thr Leu Gln
        95                  100                 105

CTG GGC TAC CCT CCA GCC CGC CCT GTT GTC TCC TGC CAA GCA GCC GAC   1105
Leu Gly Tyr Pro Pro Ala Arg Pro Val Val Ser Cys Gln Ala Ala Asp
    110                 115                 120

TAT GAG AAC TTC TCT TGC ACT TGG AGT CCC AGC CAG ATC AGC GGT TTA   1153
Tyr Glu Asn Phe Ser Cys Thr Trp Ser Pro Ser Gln Ile Ser Gly Leu
125                 130                 135                 140

CCC ACC CGC TAC CTC ACC TCC TAC AGG AAG AAG ACA GTC CTA GGA GCT   1201
Pro Thr Arg Tyr Leu Thr Ser Tyr Arg Lys Lys Thr Val Leu Gly Ala
                145                 150                 155
```

```
GAT AGC CAG AGG AGG AGT CCA TCC ACA GGG CCC TGG CCA TGC CCA CAG  1249
Asp Ser Gln Arg Arg Ser Pro Ser Thr Gly Pro Trp Pro Cys Pro Gln
        160                 165                 170

GAT CCC CTA GGG GCT GCC CGC TGT GTT GTC CAC GGG GCT GAG TTC TGG  1297
Asp Pro Leu Gly Ala Ala Arg Cys Val Val His Gly Ala Glu Phe Trp
        175                 180                 185

AGC CAG TAC CGG ATT AAT GTG ACT GAG GTG AAC CCA CTG GGT GCC AGC  1345
Ser Gln Tyr Arg Ile Asn Val Thr Glu Val Asn Pro Leu Gly Ala Ser
    190                 195                 200

ACA CGC CTG CTG GAT GTG AGC TTG CAG AGC ATC TTG CGC CCT GAC CCA  1393
Thr Arg Leu Leu Asp Val Ser Leu Gln Ser Ile Leu Arg Pro Asp Pro
205                 210                 215                 220

CCC CAG GGC CTG CGG GTA GAG TCA GTA CCA GGT TAC CCC CGA CGC CTG  1441
Pro Gln Gly Leu Arg Val Glu Ser Val Pro Gly Tyr Pro Arg Arg Leu
                225                 230                 235

CGA GCC AGC TGG ACA TAC CCT GCC TCC TGG CCG TGC CAG CCC CAC TTC  1489
Arg Ala Ser Trp Thr Tyr Pro Ala Ser Trp Pro Cys Gln Pro His Phe
        240                 245                 250

CTG CTC AAG TTC CGT TTG CAG TAC CGT CCG GCG CAG CAT CCA GCC TGG  1537
Leu Leu Lys Phe Arg Leu Gln Tyr Arg Pro Ala Gln His Pro Ala Trp
        255                 260                 265

TCC ACG GTG GAG CCA GCT GGA CTG GAG GAG GTG ATC ACA GAT GCT GTG  1585
Ser Thr Val Glu Pro Ala Gly Leu Glu Glu Val Ile Thr Asp Ala Val
    270                 275                 280

GCT GGG CTG CCC CAT GCT GTA CGA GTC AGT GCC CGG GAC TTT CTA GAT  1633
Ala Gly Leu Pro His Ala Val Arg Val Ser Ala Arg Asp Phe Leu Asp
285                 290                 295                 300

GCT GGC ACC TGG AGC ACC TGG AGC CCG GAG GCC TGG GGA ACT CCG AGC  1681
Ala Gly Thr Trp Ser Thr Trp Ser Pro Glu Ala Trp Gly Thr Pro Ser
                305                 310                 315

ACT GGG ACC ATA CCA AAG GAG ATA CCA GCA TGG GGC CAG CTA CAC ACG  1729
Thr Gly Thr Ile Pro Lys Glu Ile Pro Ala Trp Gly Gln Leu His Thr
```

```
                320                 325                 330
CAG CCA GAG GTG GAG CCT CAG GTG GAC AGC CCT GCT CCT CCA AGG CCC   1777
Gln Pro Glu Val Glu Pro Gln Val Asp Ser Pro Ala Pro Pro Arg Pro
        335                 340                 345

TCC CTC CAA CCA CAC CCT CGG CTA CTT GAT CAC AGG GAC TCT GTG GAG   1825
Ser Leu Gln Pro His Pro Arg Leu Leu Asp His Arg Asp Ser Val Glu
    350                 355                 360

CAG GTA GCT GTG CTG GCG TCT TTG GGA ATC CTT TCT TTC CTG GGA CTG   1873
Gln Val Ala Val Leu Ala Ser Leu Gly Ile Leu Ser Phe Leu Gly Leu
365                 370                 375                 380

GTG GCT GGG GCC CTG GCA CTG GGG CTC TGG CTG AGG CTG AGA CGG GGT   1921
Val Ala Gly Ala Leu Ala Leu Gly Leu Trp Leu Arg Leu Arg Arg Gly
                385                 390                 395

GGG AAG GAT GGA TCC CCA AAG CCT GGG TTC TTG GCC TCA GTG ATT CCA   1969
Gly Lys Asp Gly Ser Pro Lys Pro Gly Phe Leu Ala Ser Val Ile Pro
            400                 405                 410

GTG GAC AGG CGT CCA GGA GCT CCA AAC CTG TAGAGGACCC AGGAGGCTT     2019
Val Asp Arg Arg Pro Gly Ala Pro Asn Leu
        415                 420

CGGCAGATTC CACCTATAAT TCTGTCTTGC TGGTGTGGAT GGATGGACAG ATAGAAACCA  2079

GGCAGGACAG TAGATCCCTA TGGTTGGATC TCAGCTGGAA GTTCTGTTTG GAGCCCATTT  2139

CTGTGAGACC CTGTATTTCA AATTTGCAGC TGAAAGGTGC TTGTACCTCT GATTTCACCC  2199

CAGAGTTGGA GTTCTGCTCA AGGAACGTGT GTAATGTGTA CATCTGTGTC CATGTGTGAC  2259

CATGTGTCTG TGAGGCAGGG AACATGTATT CTCTGCATGC ATGTATGTAG GTGCCTGGGG  2319

AGTGTGTGTG GGTCCTTGGC TCTTGGCCTT TCCCCTTGCA GGGGTTGTGC AGGTGTGAAT  2379

AAAGAGAATA AGGAAGTTCT TGGAGATTAT ACTCAGAAAA AAAAAAAAAA AGTCGACGCG  2439

GCCGCGAATT CCTGCAG                                               2456
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 422 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Ser Ser Ser Cys Ser Gly Leu Ser Arg Val Leu Val Ala Val Ala
 1               5                  10                  15

Thr Ala Leu Val Ser Ala Ser Ser Pro Cys Pro Gln Ala Trp Gly Pro
            20                  25                  30

Pro Gly Val Gln Tyr Gly Gln Pro Gly Arg Ser Val Lys Leu Cys Cys
        35                  40                  45

Pro Gly Val Thr Ala Gly Asp Pro Val Ser Trp Phe Arg Asp Gly Glu
    50                  55                  60

Pro Lys Leu Leu Gln Gly Pro Asp Ser Gly Leu Gly His Glu Leu Val
65                  70                  75                  80

Leu Ala Gln Ala Asp Ser Thr Asp Glu Gly Thr Tyr Ile Cys Gln Thr
                85                  90                  95

Leu Asp Gly Ala Leu Gly Gly Thr Val Thr Leu Gln Leu Gly Tyr Pro
            100                 105                 110

Pro Ala Arg Pro Val Val Ser Cys Gln Ala Ala Asp Tyr Glu Asn Phe
        115                 120                 125

Ser Cys Thr Trp Ser Pro Ser Gln Ile Ser Gly Leu Pro Thr Arg Tyr
    130                 135                 140

Leu Thr Ser Tyr Arg Lys Lys Thr Val Leu Gly Ala Asp Ser Gln Arg
145                 150                 155                 160
```

Arg Ser Pro Ser Thr Gly Pro Trp Pro Cys Pro Gln Asp Pro Leu Gly
165 170 175

Ala Ala Arg Cys Val Val His Gly Ala Glu Phe Trp Ser Gln Tyr Arg
180 185 190

Ile Asn Val Thr Glu Val Asn Pro Leu Gly Ala Ser Thr Arg Leu Leu
195 200 205

Asp Val Ser Leu Gln Ser Ile Leu Arg Pro Asp Pro Pro Gln Gly Leu
210 215 220

Arg Val Glu Ser Val Pro Gly Tyr Pro Arg Arg Leu Arg Ala Ser Trp
225 230 235 240

Thr Tyr Pro Ala Ser Trp Pro Cys Gln Pro His Phe Leu Leu Lys Phe
245 250 255

Arg Leu Gln Tyr Arg Pro Ala Gln His Pro Ala Trp Ser Thr Val Glu
260 265 270

Pro Ala Gly Leu Glu Glu Val Ile Thr Asp Ala Val Ala Ala Gly Leu Pro
275 280 285

His Ala Val Arg Val Ser Ala Arg Asp Phe Leu Asp Ala Gly Thr Trp
290 295 300

Ser Thr Trp Ser Pro Glu Ala Trp Gly Thr Pro Ser Thr Gly Thr Ile
305 310 315 320

Pro Lys Glu Ile Pro Ala Trp Gly Gln Leu His Thr Gln Pro Glu Val
325 330 335

Glu Pro Gln Val Asp Ser Pro Ala Pro Pro Arg Pro Ser Leu Gln Pro
340 345 350

His Pro Arg Leu Leu Asp His Arg Asp Ser Val Glu Gln Val Ala Val
355 360 365

Leu Ala Ser Leu Gly Ile Leu Ser Phe Leu Gly Leu Val Ala Gly Ala
370 375 380

```
Leu Ala Leu Gly Leu Trp Leu Arg Leu Arg Arg Gly Gly Lys Asp Gly
385                 390                 395                 400

Ser Pro Lys Pro Gly Phe Leu Ala Ser Val Ile Pro Val Asp Arg Arg
                405                 410                 415

Pro Gly Ala Pro Asn Leu
            420
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1714 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 34..1359

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
TTCTTAGCCT GATAGGAGGA AGTCTTGGAG GCC ATG GCA CTC AGT CAC TGT GAT                54
                                    Met Ala Leu Ser His Cys Asp
                                    1               5

TAT CAA GAT GAG CAG CAG CTG CTC AGG GCT GAC CAG GGT CCT GGT GGC               102
Tyr Gln Asp Glu Gln Gln Leu Leu Arg Ala Asp Gln Gly Pro Gly Gly
        10                  15                  20

CGT GCT ACA GCC CTG GTG TCT TCC TCC CCC TGC CCC CAA GCT TGG                  150
Arg Ala Thr Ala Leu Val Ser Ser Ser Pro Cys Pro Gln Ala Trp
    25                  30                  35
```

```
GGT CCT CCA GGG GTC CAG TAT GGA CAA CCT GGC AGG CCC GTG ATG CTG   198
Gly Pro Pro Gly Val Gln Tyr Gly Gln Pro Gly Arg Pro Val Met Leu
 40                  45                  50                  55

TGC TGC CCC GGA GTG AGT GCT GGG ACT CCA GTG TCC TGG TTT CGG GAT   246
Cys Cys Pro Gly Val Ser Ala Gly Thr Pro Val Ser Trp Phe Arg Asp
                 60                  65                  70

GGA GAT TCA AGG CTG CTC CAG GGA CCT GAC TCT GGG TTA GGA CAC AGA   294
Gly Asp Ser Arg Leu Leu Gln Gly Pro Asp Ser Gly Leu Gly His Arg
             75                  80                  85

CTG GTC TTG GCC CAG GTG GAC AGC CCT GAT GAA GGC ACT TAT GTC TGC   342
Leu Val Leu Ala Gln Val Asp Ser Pro Asp Glu Gly Thr Tyr Val Cys
         90                  95                 100

CAG ACC CTG GAT GGT GTA TCA GGG GGC ATG GTG ACC CTG AAG CTG GGC   390
Gln Thr Leu Asp Gly Val Ser Gly Gly Met Val Thr Leu Lys Leu Gly
     105                 110                 115

TTT CCC CCA GCA CGT CCT GAA GTC TCC TGC CAA GCG GTA GAC TAT GAA   438
Phe Pro Pro Ala Arg Pro Glu Val Ser Cys Gln Ala Val Asp Tyr Glu
 120                 125                 130                 135

AAC TTC TCC TGT ACT TGG AGT CCA GGC CAG GTC AGC GGT TTG CCC ACC   486
Asn Phe Ser Cys Thr Trp Ser Pro Gly Gln Val Ser Gly Leu Pro Thr
                 140                 145                 150

CGC TAC CTT ACT TCC TAC AGG AAG AAG ACG CTG CCA GGA GCT GAG AGT   534
Arg Tyr Leu Thr Ser Tyr Arg Lys Lys Thr Leu Pro Gly Ala Glu Ser
             155                 160                 165

CAG AGG GAA AGT CCA TCC ACC GGG CCT TGG CCG TGT CCA CAG GAC CCT   582
Gln Arg Glu Ser Pro Ser Thr Gly Pro Trp Pro Cys Pro Gln Asp Pro
         170                 175                 180

CTG GAG GCC TCC CGA TGT GTG GTC CAT GGG GCA GAG TTC TGG AGT GAG   630
Leu Glu Ala Ser Arg Cys Val Val His Gly Ala Glu Phe Trp Ser Glu
     185                 190                 195
```

```
TAC CGG ATC AAT GTG ACC GAG GTG AAC CCA CTG GGT GCC AGC ACG TGC   678
Tyr Arg Ile Asn Val Thr Glu Val Asn Pro Leu Gly Ala Ser Thr Cys
200                 205                 210                 215

CTA CTG GAT GTG AGA TTA CAG AGC ATC TTG CGT CCT GAT CCA CCC CAA   726
Leu Leu Asp Val Arg Leu Gln Ser Ile Leu Arg Pro Asp Pro Pro Gln
                220                 225                 230

GGA CTG CGG GTG GAA TCC GTA CCT GGT TAC CCG AGA CGC CTG CAT GCC   774
Gly Leu Arg Val Glu Ser Val Pro Gly Tyr Pro Arg Arg Leu His Ala
            235                 240                 245

AGC TGG ACA TAC CCT GCC TCC TGG CGT CGC CAA CCC CAC TTT CTG CTC   822
Ser Trp Thr Tyr Pro Ala Ser Trp Arg Arg Gln Pro His Phe Leu Leu
        250                 255                 260

AAG TTC CGG TTG CAA TAC CGA CCA GCA CAG CAT CCA GCC TGG TCC ACG   870
Lys Phe Arg Leu Gln Tyr Arg Pro Ala Gln His Pro Ala Trp Ser Thr
    265                 270                 275

GTG GAG CCC ATT GGC TTG GAG GAA GTG ATA ACA GAT GCT GTG GCT GGG   918
Val Glu Pro Ile Gly Leu Glu Glu Val Ile Thr Asp Ala Val Ala Gly
280                 285                 290                 295

CTG CCA CAC GCG GTA CGA GTC AGT GCC AGG GAC TTT CTG GAT GCT GGC   966
Leu Pro His Ala Val Arg Val Ser Ala Arg Asp Phe Leu Asp Ala Gly
                300                 305                 310

ACC TGG AGC GCC TGG AGC CCA GAG GCC TGG GGT ACT CCT AGC ACT GGT  1014
Thr Trp Ser Ala Trp Ser Pro Glu Ala Trp Gly Thr Pro Ser Thr Gly
            315                 320                 325

CCC CTG CAG GAT GAG ATA CCT GAT TGG AGC CAG GGA CAT GGA CAG CAG  1062
Pro Leu Gln Asp Glu Ile Pro Asp Trp Ser Gln Gly His Gly Gln Gln
        330                 335                 340

CTA GAG GCA GTA GCT CAG GAG GAC AGC CCG GCT CCT GCA AGG CCT      1110
Leu Glu Ala Val Ala Gln Glu Asp Ser Pro Ala Pro Ala Arg Pro
    345                 350                 355

TCC TTG CAG CCG GAC CCA AGG CCA CTT GAT CAC AGG GAC CCC TTG GAG  1158
Ser Leu Gln Pro Asp Pro Arg Pro Leu Asp His Arg Asp Pro Leu Glu
```

```
360                 365                 370                 375

CAA GTA GCT GTG TTA GCG TCT CTG GGA ATC TTC TCT TGC CTT GGC CTG      1206
Gln Val Ala Val Leu Ala Ser Leu Gly Ile Phe Ser Cys Leu Gly Leu
                380                 385                 390

GCT GTT GGA GCT CTG GCA CTG GGG CTC TGG CTG AGG CTG AGA CGG AGT      1254
Ala Val Gly Ala Leu Ala Leu Gly Leu Trp Leu Arg Leu Arg Arg Ser
            395                 400                 405

GGG AAG GAT GGA CCG CAA AAA CCT GGG CTC TTG GCA CCC ATG ATC CCG      1302
Gly Lys Asp Gly Pro Gln Lys Pro Gly Leu Leu Ala Pro Met Ile Pro
        410                 415                 420

GTG GAA AAG CTT CCA GGA ATT CCA AAC CTG CAG AGG ACC CCA GAG AAC      1350
Val Glu Lys Leu Pro Gly Ile Pro Asn Leu Gln Arg Thr Pro Glu Asn
    425                 430                 435

TTC AGC TGATTTCATC TGTAACCCGG TCAGACTTGG GGTGGTTAAA AGGACAGGCA      1406
Phe Ser
440

GAAAGAGGCG GGGCAGTGGA TCCCTGTGGA TGGAGGTCTC AGCTGAAAGT CTGAGCTCTT   1466

TTCTTTGACA CCTATACTCC AAACTTGCTG CCGGCTGAAG GCTGTCTGGA CTTCCGATGT   1526

CCTGAGGTGG AAGTCCACCT GAGGAATGTG TACAGAAGTC TGTGTTCCTG TGATCGTGTG   1586

TGTATGTGAG ACAGGGAGCA AAAGTTCTCT GCATGTGTGT ACAGATGATT GGAGAGTGTG   1646

TGCGGTCTTG GGCTTGGCCC TTCTGGGAAG TGTGAAGAGT TGAAATAAAA GAGACGGAAG   1706

TTTTTGGA                                                            1714
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 441 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Ala Leu Ser His Cys Asp Tyr Gln Asp Glu Gln Gln Leu Leu Leu Arg
 1               5                  10                  15

Ala Asp Gln Gly Pro Gly Gly Arg Ala Thr Ala Leu Val Ser Ser Ser Ser
            20                  25                  30

Ser Pro Cys Pro Gln Ala Trp Gly Pro Pro Gly Val Gln Tyr Gly Gln Gln
        35                  40                  45

Pro Gly Arg Pro Val Met Leu Cys Cys Pro Gly Val Ser Ala Gly Thr Thr
    50                  55                  60

Pro Val Ser Trp Phe Arg Asp Gly Asp Ser Arg Leu Leu Gln Gly Pro Pro
65                  70                  75                  80

Asp Ser Gly Leu Gly His Arg Leu Val Leu Ala Gln Val Asp Ser Pro Pro
                85                  90                  95

Asp Glu Gly Thr Tyr Val Cys Gln Thr Leu Asp Gly Val Ser Gly Gly Gly
            100                 105                 110

Met Val Thr Leu Lys Leu Gly Phe Pro Pro Ala Arg Pro Glu Val Ser Ser
        115                 120                 125

Cys Gln Ala Val Asp Tyr Glu Asn Phe Ser Cys Thr Trp Ser Pro Gly Gly
    130                 135                 140

Gln Val Ser Gly Leu Pro Thr Arg Tyr Leu Thr Ser Tyr Arg Lys Lys Lys
145                 150                 155                 160

Thr Leu Pro Gly Ala Glu Ser Gln Arg Glu Ser Pro Ser Thr Gly Pro Pro
                165                 170                 175

Trp Pro Cys Pro Gln Asp Pro Leu Glu Ala Ser Arg Cys Val Val His His
            180                 185                 190

Gly Ala Glu Phe Trp Ser Glu Tyr Arg Ile Asn Val Thr Glu Val Asn Asn
        195                 200                 205
```

Pro Leu Gly Ala Ser Thr Cys Leu Leu Asp Val Arg Leu Gln Ser Ile
210 215 220

Leu Arg Pro Asp Pro Pro Gln Gly Leu Arg Val Glu Ser Val Pro Gly
225 230 235 240

Tyr Pro Arg Arg Leu His Ala Ser Trp Thr Tyr Pro Ala Ser Trp Arg
245 250 255

Arg Gln Pro His Phe Leu Leu Lys Phe Arg Leu Gln Tyr Arg Pro Ala
260 265 270

Gln His Pro Ala Trp Ser Thr Val Glu Pro Ile Gly Leu Glu Glu Val
275 280 285

Ile Thr Asp Ala Val Ala Gly Leu Pro His Ala Val Arg Val Ser Ala
290 295 300

Arg Asp Phe Leu Asp Ala Gly Thr Trp Ser Ala Trp Ser Pro Glu Ala
305 310 315 320

Trp Gly Thr Pro Ser Thr Gly Pro Leu Gln Asp Glu Ile Pro Asp Trp
325 330 335

Ser Gln Gly His Gly Gln Leu Glu Ala Val Val Ala Gln Glu Asp
340 345 350

Ser Pro Ala Pro Ala Arg Pro Ser Leu Gln Pro Asp Pro Arg Pro Leu
355 360 365

Asp His Arg Asp Pro Leu Glu Gln Val Ala Val Leu Ala Ser Leu Gly
370 375 380

Ile Phe Ser Cys Leu Gly Leu Ala Val Gly Ala Leu Ala Leu Gly Leu
385 390 395 400

Trp Leu Arg Leu Arg Arg Ser Gly Lys Asp Gly Pro Gln Lys Pro Gly
405 410 415

Leu Leu Ala Pro Met Ile Pro Val Glu Lys Leu Pro Gly Ile Pro Asn
420 425 430

Leu Gln Arg Thr Pro Glu Asn Phe Ser
435 440

What is claimed is:

1. An isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence of SEQ ID NO:1 from nucleotide 803 to nucleotide 1999;

(b) a nucleotide sequence varying from the sequence of the nucleotide sequence specified in (a) as a result of degeneracy of the genetic code; and

(c) an allelic variant of the nucleotide sequence specified in (a).

2. The polynucleotide of claim 1 wherein said nucleotide sequence encodes for a protein having a biological activity of the human IL-11 receptor.

3. The polynucleotide of claim 1 wherein said nucleotide sequence is operably linked to an expression control sequence.

4. The polynucleotide of claim 2 comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 803 to nucleotide 1999.

5. The polynucleotide of claim 2 comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 803 to nucleotide 1828 or a fragment thereof.

6. The polynucleotide of claim 2 comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 1907 to nucleotide 1999 or a fragment thereof.

7. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 734 to nucleotide 1999.

8. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 1067 to nucleotide 1828.

9. The polynucleotide of claim 1 comprising the nucleotide sequence of SEQ ID NO:1 from nucleotide 1067 to nucleotide 1999.

10. A host cell transformed with the polynucleotide of claim 3.

11. The host cell of claim 8, wherein said cell is a mammalian cell.

12. A process for producing a human IL-11R protein, said process comprising:

(a) growing a culture of the host cell of claim 10 in a suitable culture medium; and

(b) purifying the human IL-11R protein from the culture.

13. An isolated human IL-11R protein comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2;

(b) the amino acid sequence of SEQ ID NO:2 from amino acids 24 to 422;

(c) the amino acid sequence of SEQ ID NO:2 from amino acids 24 to 365;

(d) the amino acid sequence of SEQ ID NO:2 from amino acids 391 to 422;

(e) the amino acid sequence of SEQ ID NO:2 from amino acids 102 to 422;

(f) the amino acid sequence of SEQ ID NO:2 from amino acids 102 to 365; and

(g) fragments of (a)-(f) having a biological activity of the human IL-11 receptor.

14. The protein of claim 13 comprising the amino acid sequence of SEQ ID NO:2.

15. The protein of claim 13 comprising the sequence from amino acid 24 to 365 of SEQ ID NO:2.

16. A pharmaceutical composition comprising a protein of claim 13 and a pharmaceutically acceptable carrier.

17. A protein produced according to the process of claim 12.

18. A composition comprising an antibody which specifically reacts with a protein of claim 13.

19. A method of identifying an inhibitor of IL-11 binding to the human IL-11 receptor which comprises:

(a) combining a protein of claim 13 with IL-11 or a fragment thereof, said combination forming a first binding mixture;

(b) measuring the amount of binding between the protein and the IL-11 or fragment in the first binding mixture;

(c) combining a compound with the protein and the IL-11 or fragment to form a second binding mixture;

(d) measuring the amount of binding in the second binding mixture; and

(e) comparing the amount of binding in the first binding mixture with the amount of binding in the second binding mixture;

wherein the compound is capable of inhibiting IL-11 binding to the human IL-11 receptor when a decrease in the amount of binding of the second binding mixture occurs.

20. The method of claim 19 wherein the first and second binding mixture comprise gp130 or a fragment thereof capable of binding to the protein of claim 13 or the IL-11 or fragment used therein.

21. An inhibitor identified by the method of claim 19.

22. A pharmaceutical composition comprising the inhibitor of claim 21 and a pharmaceutically acceptable carrier.

23. A method of inhibiting binding of IL-11 to the human IL-11 receptor in a mammalian subject, said method comprising administering a therapeutically effective amount of a composition of claim 22.

24. A method of inhibiting binding of IL-11 to the human IL-11 receptor in a mammalian subject, said method comprising administering a therapeutically effective amount of a composition of claim 16.

25. A method of inhibiting binding of IL-11 to the human IL-11 receptor in a mammalian subject, said method comprising administering a therapeutically effective amount of a composition of claim 18.

26. A method of treating or preventing loss of bone mass in a mammalian subject, said method comprising administering a therapeutically effective amount of a composition of claim 22.

27. A method of treating or preventing loss of bone mass in a mammalian subject, said method comprising administering a therapeutically effective amount of a composition of claim 16.

28. A method of treating or preventing loss of bone mass in a mammalian subject, said method comprising administering a therapeutically effective amount of a composition of claim 18.

29. An isolated polynucleotide comprising a nucleotide sequence capable of hybridizing under stringent conditions to polynucleotide of claim 4.

30. An isolated polynucleotide comprising a nucleotide sequence encoding a peptide or protein comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2;

(b) the amino acid sequence of SEQ ID NO:2 from amino acids 24 to 422;

(c) the amino acid sequence of SEQ ID NO:2 from amino acids 24 to 365;

(d) the amino acid sequence of SEQ ID NO:2 from amino acids 391 to 422;

(e) the amino acid sequence of SEQ ID NO:2 from amino acids 112 to 422;

(f) the amino acid sequence of SEQ ID NO:2 from amino acids 112 to 365; and

(g) fragments of (a)-(f) having a biological activity of the human IL-11 receptor.

FIG. 1/1

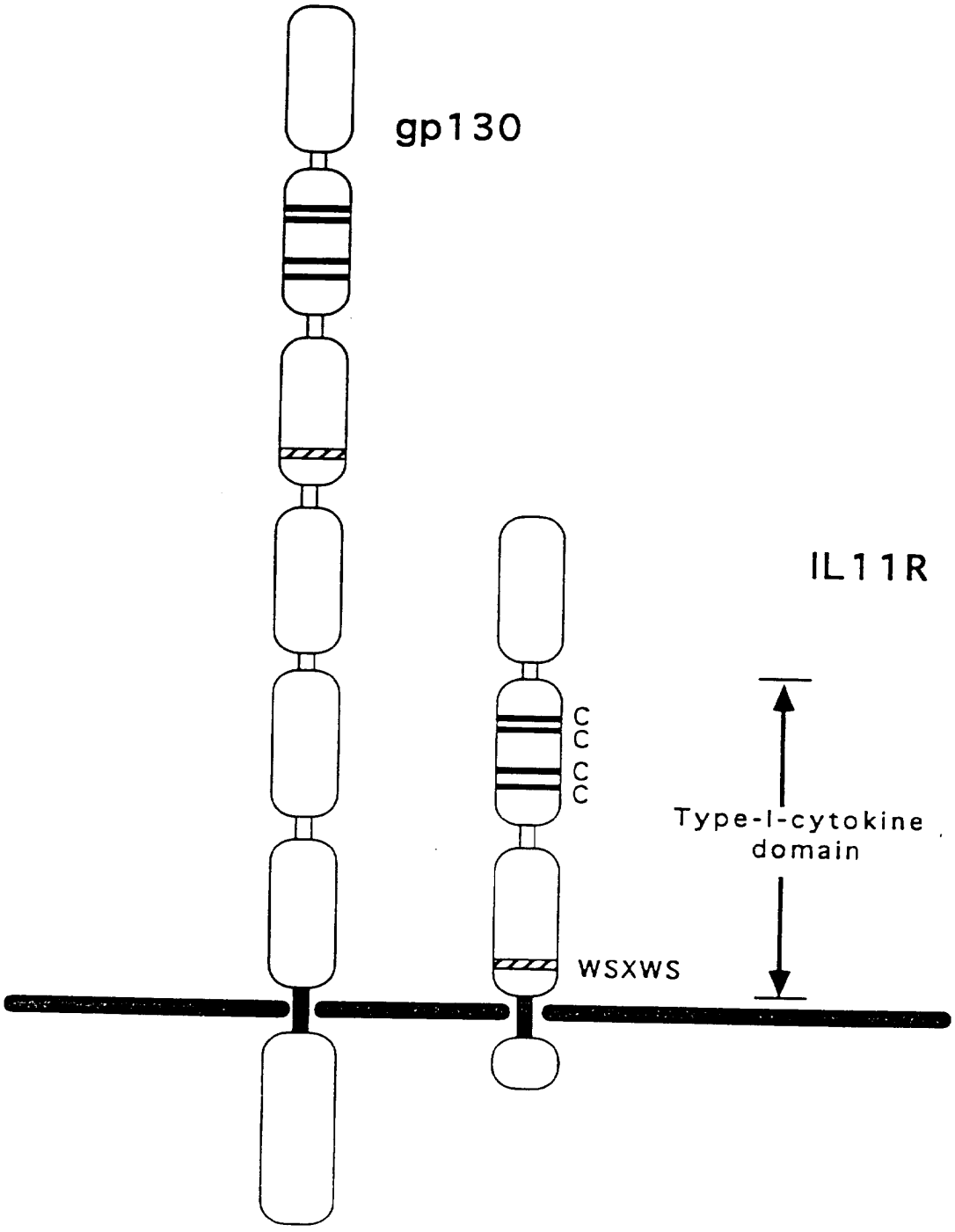
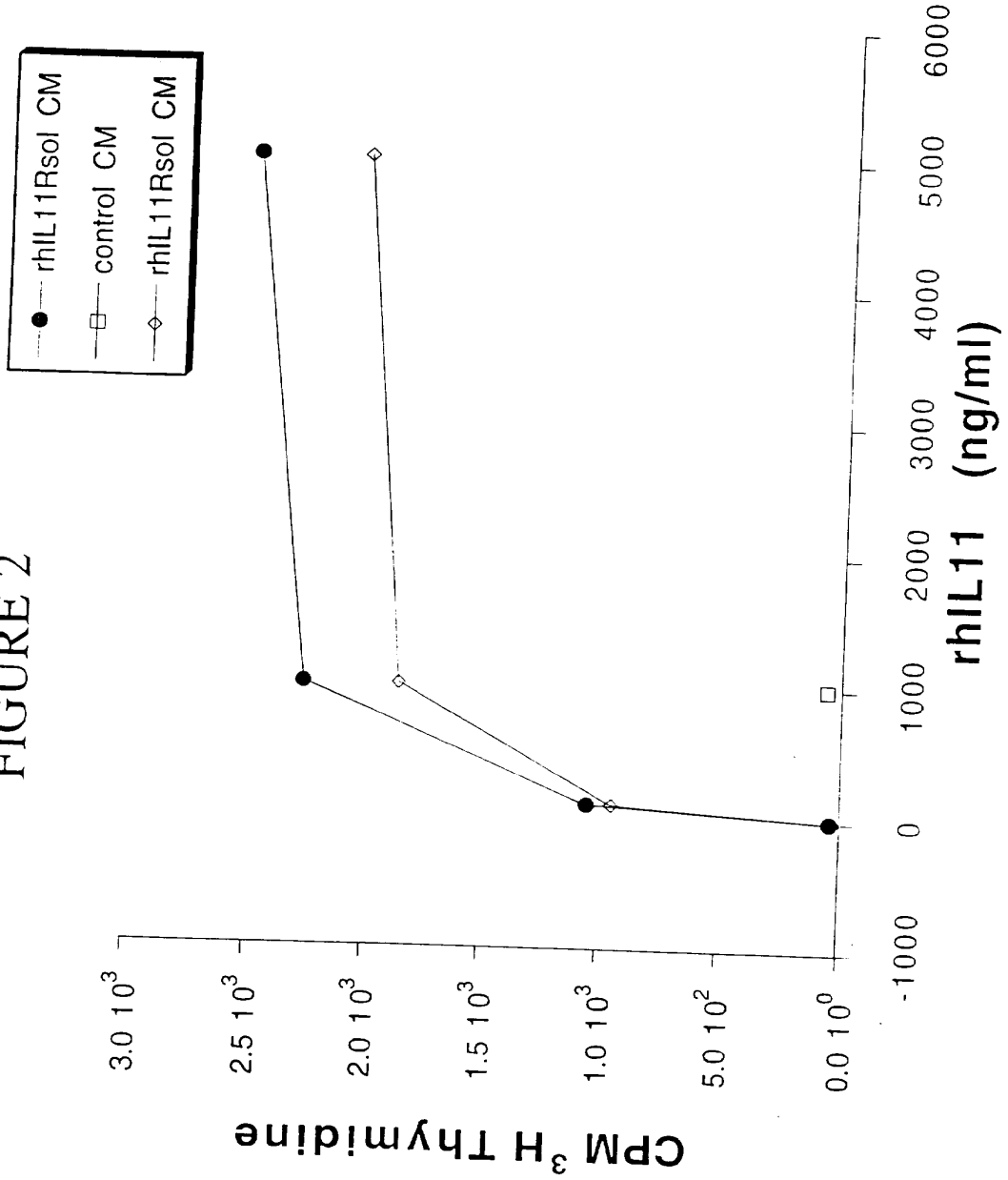


FIGURE 2


rhIL11Rsol CM
control CM
rhIL11Rsol CM
CPM ³H Thymidine
3.0 10³
2.5 10³
2.0 10³
1.5 10³
1.0 10³
5.0 10²
0.0 10⁰
-1000
0
1000
2000
3000
4000
5000
6000
rhIL11 (ng/ml)

INTERNATIONAL SEARCH REPORT

rnational Application No
PCT/US 95/15400

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/715 A61K38/17 C07K16/28 C12N5/10
A61K39/395 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DEVELOPMENTAL BIOLOGY, vol. 166, 1994, pages 531-542, XP002000295 H. NEUHAUS ET AL: "Etl2, a novel putative type-I cytokine receptor expressed during mouse embryogenesis at high levels in skin and cells with skeletogenic potential" *see the whole document especially figure 3 page 535 * --- -/--	1,4-9, 29,30

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
3 April 1996	23. 04. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Le Cornec, N

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ernational Application No
PCT/US 95/15400

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBO JOURNAL, vol. 13, no. 20, 17 October 1994, EYNSHAM, OXFORD GB, pages 4765-4775, XP002000296 D.J. HILTON ET AL: "Cloning of a murine IL-11 receptor alpha-chain ; requirement for gp130 for high affinity binding and signal transduction" see page 4766, right-hand column, line 9 - line 37 see page 4769, right-hand column, line 17 - line 20 see figure 1	1,4-9, 29,30
P,X	BLOOD, vol. 86, no. 7, 1 October 1995, pages 2534-2540, XP002000297 M. CHEREL ET AL: "Molecular cloning of two isoforms of a receptor for the human hematopoietic cytokine Interleukin-11" see the whole document	1,2,4-9, 29,30
A	EUROPEAN JOURNAL OF IMMUNOLOGY , vol. 24, no. 1, January 1994, pages 277-280, XP002000298 M. FOURCIN ET AL: "Involvement of gp130/interleukin-6 receptor transducing component in Interleukin-11 receptor" see page 278, right-hand column - page 279	19,20
A	BIOFACTORS, vol. 4, no. 1, December 1992, pages 15-21, XP002000299 YU-CHUNG YANG ET AL: "Interleukin-11 and its receptor" see page 17 - page 18	13-15,19

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/12, C07K 14/715, A61K 38/17, C07K 16/28, C12P 21/00

A2

(11) International Publication Number: **WO 99/20755**

(43) International Publication Date: 29 April 1999 (29.04.99)

(21) International Application Number: PCT/EP98/06497

(22) International Filing Date: 14 October 1998 (14.10.98)

(30) Priority Data:
9721961.2 16 October 1997 (16.10.97) GB

(71) Applicant *(for all designated States except US)*: GLAXO GROUP LIMITED [GB/GB]; Glaxo Wellcome House, Berkeley Avenue, Greenford, Middlesex UB6 0NN (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ELSON, Greg [GB/FR]; Centre d'Immunologie Pierre Fabre, 5, avenue Napoléon III, F–74164 Saint Julien en Genevois (FR). GAUCHAT, Jean–François [CH/FR]; Centre d'Immunologie Pierre Fabre, 5, avenue Napoléon III, F–74164 Saint Julien en Genevois (FR). KOSCO–VILBOIS, Marie [US/CH]; Serono Pharmaceutical Research Institute S.A., 14, chemin des Aulx, CH–1228 Plan–les–Ouates (CH).

(74) Agent: TEUTEN, Andrew, J.; Glaxo Wellcome plc, Glaxo Wellcome House, Berkeley Avenue, Greenford, Middlesex UB6 0NN (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: NOVEL CYTOKINE RECEPTORS

(57) Abstract

A novel polypeptide that is believed to be a novel type I cytokine receptor has been identified in both mice and in humans and the corresponding cDNA sequences have been obtained. There is a high degree of conservation of amino acid between the human and murine polypeptides, indicating that this receptor is functionally important. Polypeptides within the scope of the present invention may be useful in treating cancer, obesity and immune or developmental disorders. They may also be useful in screening.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Novel Cytokine Receptors

The present invention relates *inter alia* to novel molecules believed to be cytokine receptors and to uses thereof.

Cytokines and growth factors are secreted molecules controlling important cell functions such as proliferation, differentiation and survival as well as tissue development. These signalling molecules exert their effects via specific receptors located on the target cell surface. These receptors are grouped into families according to both structural and amino acid sequence similarities. The cytokine receptor superfamily is composed of the receptors for many growth factor families including interferon, TNF and haematopoietic growth factors. The largest subclass in this family is that of the type I cytokine receptors, a group characterized by the presence of a conserved extracellular region of approximately 200 amino acids containing two fibronectin type III folds. This region, known as the haematopoietin receptor module, has been shown to play an essential role in receptor/ligand binding and receptor/receptor dimerization. It is characterized by four conserved cysteine residues in the first domain and a W-S-x-W-S motif in the second domain.

Receptor chains in this subclass typically form part of a multicomponent complex which includes both ligand binding and signalling subunits, of which the latter is typically a member of several receptor complexes. These characteristics account for much of the pleitropy and redundancy amongst cytokines. One such example is amongst certain members of the interleukin-6 (IL-6) related cytokines (IL-6, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM) and cardiotrophin-1 (CT-1)), where overlapping function between these cytokines can be accounted for by the presence of gp130, a signal transducing molecule present in the respective receptor complexes. In addition, ligand binding chains in the receptor complexes (such as IL-6Rα, CNTF-R and IL-11Rα) generally have short or even absent intracytoplasmic

regions, whereas gp130 has a longer intracytoplasmic tail which is involved in the activation of the JAK-STAT pathway. In fact, soluble forms of these receptor subunits can render cells sensitive to the appropriate cytokine provided the necessary signal transducing chain is expressed on the cell surface. Thus, the specific cytokine binding chains of these cytokine receptors can function either as membrane anchored or soluble proteins.

The present invention is based upon the identification and characterisation of a previously unknown mouse molecule and of a previously unknown human molecule, both of which are believed to be new type 1 cytokine receptors.

According to the present invention there is provided a polypeptide, which:

a) has the amino acid sequence of amino acids 38 to 422 shown in Figure 1 for hGBRI-ILR or which has the amino acid sequence shown in Figure 1 for mGBRI-ILR;

b) has one or more amino acid deletions, insertions or substitutions relative to a polypeptide as defined in a) above, but has at least 40% amino acid sequence identity therewith; or

c) is a fragment of a polypeptide as defined in a) or b) above, which is at least 10 amino acids long.

The term "polypeptide" is used herein in a broad sense to indicate that a particular molecule comprises a plurality of amino acids joined together by peptide bonds. It therefore includes within its scope substances which may sometimes be referred to in the literature as peptides, polypeptides or proteins.

A polypeptide of the present invention preferably incorporates a haematopoietin domain since this is believed to be important in ligand / receptor and receptor / receptor complex formation.

Various aspects of the present invention will now be considered in order that its full scope can be appreciated.

Polypeptides of the present invention may be produced by techniques known to those skilled in the art. For example, gene-cloning techniques may be used to provide a nucleic acid sequence encoding such a polypeptide. (Gene-cloning techniques are discussed in greater detail later on in relation to nucleic acid molecules of the present invention.) Alternatively, chemical synthesis techniques may be used to produce polypeptides of the present invention. Such techniques generally utilise solid-phase synthesis. Chemical synthesis techniques that allow polypeptides having particular sequences to be produced have now been automated. Apparatuses capable of chemically synthesising polypeptides are available, for example, from Applied Biosystems. If desired, short polypeptides can be synthesised initially and can then be ligated to produce longer polypeptides.

A polypeptide of the present invention may be provided in substantially pure form. Thus it may be provided in a composition in which it is the predominant polypeptide component present. (It may be present e.g. at a level of more than 50%, of more than 75%, of more than 90%, or even of more than 95%; said levels being determined on a weight/weight basis with respect to the total polypeptide content of the composition.)

As explained previously, a polypeptide of the present invention either:

a) has the amino acid sequence of amino acids 38 to 422 shown in Figure 1 for hGBRI-ILR or has the amino acid sequence shown in Figure 1 for mGBRI-ILR;

b) has one or more amino acid deletions, insertions or substitutions relative to a polypeptide as defined in a) above, but has at least 40% amino acid sequence identity therewith; or

c) is a fragment of a polypeptide as defined in a) or b) above, which is at least 10 amino acids long.

In order to appreciate the present invention more fully, polypeptides within the scope of each of a), b) and c) above will now each be discussed in greater detail.

Polypeptides within the scope of a)

A polypeptide within the scope of a) may consist of the amino acid sequence of amino acids 38 to 422 shown in Figure 1 for hGBRI-ILR or of the amino acid sequence shown in Figure 1 for mGBRI-ILR. Alternatively it may have an additional N-terminal and/or an additional C-terminal amino acid sequence.

Additional N-terminal or C-terminal sequences may be provided for various reasons. Techniques for providing such additional sequences are well known in the art. These include using gene-cloning techniques to ligate together nucleic acid molecules encoding polypeptides or parts thereof, followed by expressing a polypeptide encoded by the nucleic acid molecule produced by ligation.

Additional sequences may be provided in order to alter the characteristics of a particular polypeptide. This can be useful in improving expression or regulation of expression in particular expression systems. For example, an additional sequence may provide some protection against proteolytic cleavage. This has been done for the hormone somatostatin by fusing it at its N-terminus to part of the β galactosidase enzyme (Itakwa *et al.*, *Science* **198**: 105-63 (1977)).

Additional sequences can also be useful in altering the properties of a polypeptide to aid in directing the polypeptide to a particular location. For example, a signal sequence may be present to direct the transport of the polypeptide to a particular location within a cell or to export the polypeptide from the cell (e.g. the amino acids 1 to 37 shown in Figure

1 for hGBRI-ILR may be used to provide a signal sequence, or another signal sequence may be present). Different signal sequences can be used for different expression systems.

Hydrophobic sequences may be provided to anchor a polypeptide in a membrane. Thus the present invention includes within its scope both soluble and membrane-bound polypeptides. (Naturally occurring membrane-bound forms of the polypeptides identified in Figure 1 are believed to exist since large mRNA transcripts likely to encode such forms have been identified by the present inventors, as will be discussed later.) Membrane-bound polypeptides may be in hybrid form if desired. They may therefore have a heterologous transmembrane and/or cytoplasmic domain. For example such domains may be derived from a human IL-13 receptor α chain. Transfected mammalian cells expressing membrane-bound polypeptides can be used for ligand screening and binding assays (e.g. for antibodies or other molecules binding to the receptor).

Another example of the provision of an additional sequence is where a polypeptide is linked to a moiety aiding in purification / identification, e.g. a moiety capable of being isolated by affinity chromatography. The moiety may be an epitope and the affinity column may comprise immobilised antibodies or immobilised antibody fragments that bind to said epitope (desirably with a high degree of specificity). The polypeptide can then be eluted from the column by addition of an appropriate buffer and may be cleaved from the epitope. His_6, Glu_2 or 179 tags are preferred for use in purification / identification. Polypeptides comprising one or more such tags are therefore within the scope of the present invention.

A polypeptide may be linked to an antibody or to a part thereof. For example it may be linked to an F_c portion. This results in a molecule with good stability that can be used both *in vitro* and *in vivo*. It may be linked to a part of an antibody that binds to a particular epitope of it is desired to target that epitope.

In the case of the of the amino acid sequence shown in Figure 1 for mGBRI-ILR, additional amino acids may be provided to result in an amino acid sequence closer in length to the length of the 422 amino acid polypeptide shown in Figure 1 for hGBRI-ILR. For example, an additional two amino acids immediately N-terminal to the amino acid sequence shown in Figure 1 for mGBRI-ILR polypeptide may be provided. (These may be A and H, as is the case for hGBRI-ILR). A signal sequence may also/alternatively be provided.

It should be noted that additional N-terminal or C-terminal sequences may be present simply as a result of a particular technique used to obtain a polypeptide of the present invention and need not provide any particular advantageous characteristic.

Polypeptides within the scope of b)

Turning now to the polypeptides defined in b) above, it will be appreciated by a person skilled in the art that these are variants of the polypeptides given in a) above.

The skilled person will appreciate that various changes can sometimes be made to the amino acid sequence of a polypeptide which has a desired property to produce variants (often known as "muteins") that still have said property. Such variants of the polypeptides described in a) above are within the scope of the present invention and are discussed in greater detail below in sections *(i)* to *(iii)*. They include allelic and non-allelic variants.

(i) Substitutions

An example of a variant of the present invention is a polypeptide as defined in a) above, apart from the substitution of one or more amino acids with one or more other amino acids.

The skilled person is aware that various amino acids have similar characteristics. One or more such amino acids of a polypeptide can often be substituted by one or more other such amino acids without eliminating a desired property of that polypeptide.

For example, the amino acids glycine, alanine, valine, leucine and isoleucine can often be substituted for one another (amino acids having aliphatic side chains). Of these possible substitutions it is preferred that glycine and alanine are used to substitute for one another (since they have relatively short side chains) and that valine, leucine and isoleucine are used to substitute for one another (since they have larger aliphatic side chains which are hydrophobic).

Other amino acids that can often be substituted for one another include:
phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains);
lysine, arginine and histidine (amino acids having basic side chains);
aspartate and glutamate (amino acids having acidic side chains);
asparagine and glutamine (amino acids having amide side chains);
and cysteine and methionine (amino acids having sulphur containing side chains).

Substitutions of this nature are often referred to as "conservative" or "semi-conservative" amino acid substitutions.

(ii)Deletions

Amino acid deletions can be advantageous since the overall length and the molecular weight of a polypeptide can be reduced whilst still retaining a desired property. This can enable the amount of polypeptide required for a particular purpose to be reduced. For example if the polypeptide is to be used in medicine, dosage levels can be reduced.

(iii) Insertions

Amino acid insertions relative to a polypeptide as defined in a) above can also be made. This may be done to alter the nature of the polypeptide (e.g. to assist in identification, purification or expression, as explained above in relation to fusion proteins).

Polypeptides incorporating amino acid changes (whether substitutions, deletions or insertions) relative to the sequence of a polypeptide as defined in a) above can be provided using any suitable techniques. For example, a nucleic acid sequence incorporating a desired sequence change can be provided by site-directed mutagenesis. This can then be used to allow the expression of a polypeptide having a corresponding change in its amino acid sequence.

Whatever amino acid changes may be made, preferred polypeptides of the present invention have at least 40% amino acid sequence identity with the amino acid sequence of amino acids 38 to 422 shown in Figure 1 for hGBRI-ILR or with the amino acid sequence shown in Figure 1 for mGBRI-ILR. More preferably the degree of sequence identity is at least 50% or at least 75%. Sequence identities of at least 90% or of at least 95% are most preferred.

For the purposes of the present invention sequence identity (whether amino acid or nucleic acid) can be determined by using the "BESTFIT" program of the Wisconsin Sequence Analysis Package Genetics Computer Group version 8.0.

Where high degrees of sequence identity are present there may be relatively few differences in amino acid sequence. Thus for example there may be less than 20, less than 10, or even less than 5 differences.

Polypeptides within the scope of c)

As discussed *supra*, it is often advantageous to reduce the length of a polypeptide. Feature c) of the present invention therefore covers fragments of the polypeptides a) or

b) above which are at least 10 amino acids long. Desirably these fragments are at least 20, at least 50 or at least 100 amino acids long. Fragments may be useful, for example, in raising antibodies against particular antigens. They may also be useful in studying functionally important domains of a full length polypeptide. Thus, for example, a fragment comprising all or part of a haematopoietin receptor module may be provided.

Uses of Polypeptides

A) Medical Uses

Polypeptides of the present invention may be used in medicine. Preferred treatments are human treatments, although veterinary treatments are not excluded. The treatment may be prophylactic or may be in respect of an existing condition.

Various polypeptides of the present invention may be useful either as agonists or as antagonists. Agonists will up-regulate a biological function of a naturally occurring receptor, whereas antagonists will down-regulate such a function. Whether or not a given polypeptide acts as an agonist or an antagonist of a particular biological function can be determined by a skilled person using an appropriate assay procedure.

Antagonists may be useful in treating disorders associated with an overexpression of a cytokine or with the expression of a moiety having a level of cytokine activity higher than normal. In view of the homology with IL6-R, antagonists of one or more of the functions of receptors of the present invention may be useful in treating disorders associated with high levels of cell proliferation (e.g. in treating cancer). Antagonists may also be useful in treating immune disorders, weight disorders and / or developmental disorders. In particular they may be useful in treating obesity (in view of homology of the polypeptides shown in Figure 1 with the leptin receptor), inflammation, septic shock, AIDS and disorders of embryonic development.

Agonists may be useful in treating disorders associated with an underexpression of a cytokine or with the expression of a moiety having a level of cytokine activity lower than normal. They may be useful in treating disorders associated with low levels of cell proliferation. Agonists may also be useful in treating immune disorders, weight disorders and / or developmental disorders. In particular they may be useful in treating obesity (in view of homology of the polypeptides shown in Figure 1 with the leptin receptor), inflammation, septic shock, AIDS and disorders of embryonic development.

Antagonists or agonists may therefore be used in the manufacture of a medicament for the treatments mentioned above.

The medicament will usually be supplied as part of a pharmaceutical composition, which may include a pharmaceutically acceptable carrier. This pharmaceutical composition will generally be provided in a sterile form in a sealed container. It may be provided in unit dosage form, will generally be provided in a sealed container, and can be provided as part of a kit. Such a kit is within the scope of the present invention. It would normally (although not necessarily) include instructions for use. A plurality of unit dosage forms may be provided.

Pharmaceutical compositions within the scope of the present invention may include one or more of the following: preserving agents, solubilising agents, stabilising agents, wetting agents, emulsifiers, sweeteners, colorants, odourants, salts, buffers, coating agents or antioxidants. They may also contain therapeutically active agents in addition to polypeptides of the present invention. They may be provided in controlled release form, e.g. so as to be effective over a period of at least a week or, more preferably, of at least a month.

A pharmaceutical composition within the scope of the present invention may be adapted for administration by any appropriate route, for example by the oral (including buccal or

sublingual), rectal, nasal, topical (including buccal, sublingual or transdermal), vaginal or parenteral (including subcutaneous, intramuscular, intravenous or intradermal) routes. Such a composition may be prepared by any method known in the art of pharmacy, for example by admixing one or more active ingredients with a suitable carrier under sterile conditions.

Dosages

Dosages of an active agent can vary between wide limits, depending upon the nature of the treatment, the age and condition of the individual to be treated, etc. and a physician will ultimately determine appropriate dosages to be used. A dosage may be repeated as often as appropriate. If side effects develop, the amount and/or frequency of the dosage can be reduced, in accordance with good clinical practice.

B) Diagnostics Uses

In addition to the medical uses discussed above, polypeptides of the present invention can be used in diagnosis. For example they can be used in binding studies to diagnose the presence or absence of a type 1 cytokine or to diagnose abnormalities in the level of such a cytokine.

C) Screening Uses

Polypeptides of the present invention can also be used in screening. For example soluble or membrane bound receptors / variants thereof may be used to screen for agents capable of binding thereto. Such agents may be the cytokines which normally bind to the receptors *in vivo*. Alternatively they may be agonists or antagonists of such cytokines and may be useful in treating one or more of the disorders discussed in A) above.

D) Uses in Raising or Selecting Antibodies

One further use of the polypeptides of the present invention is in raising or selecting antibodies.

The present invention therefore includes antibodies that bind to a polypeptide of the present invention. Preferred antibodies bind specifically to polypeptides of the present invention and can therefore be used to purify such polypeptides (e.g. they may be immobilised and used to bind to polypeptides of the present invention. The polypeptides may then be eluted by washing with a suitable eluent under appropriate conditions.)

Antibodies within the scope of the present invention may be monoclonal or polyclonal.

Polyclonal antibodies can be raised by stimulating their production in a suitable animal host (e.g. a mouse, rat, guinea pig, rabbit, sheep, goat or monkey) when a polypeptide of the present invention or a nucleic acid molecule (e.g. cDNA) capable of being used to provide such a polypeptide is injected into the animal. If necessary an adjuvant may be administered together with the polypeptide of the present invention. The antibodies can then be purified by virtue of their binding to the polypeptide.

Monoclonal antibodies can be produced from hybridomas. These can be formed by fusing myeloma cells and spleen cells which produce the desired antibody in order to form an immortal cell line. Thus the well-known Kohler & Milstein technique (*Nature* **256** 52-55 (1975)) or variations upon this technique can be used.

Techniques for producing monoclonal and polyclonal antibodies which bind to a particular polypeptide are now well developed in the art. They are discussed in standard immunology textbooks, for example in Roitt *et al*, *Immunology* second edition (1989), Churchill Livingstone, London.

In addition to whole antibodies, the present invention includes derivatives thereof which are capable of binding to polypeptides of the present invention. Thus the present invention includes antibody fragments and synthetic constructs. Examples of antibody

fragments and synthetic constructs are given by Dougall *et al* in *Tibtech* **12** 372-379 (September 1994).

Antibody fragments include, for example, Fab, $F(ab')_2$ and Fv fragments (these are discussed in Roitt *et al* [*supra*], for example). Fv fragments can be modified to produce a synthetic construct known as a single chain Fv (scFv) molecule. This includes a peptide linker covalently joining V_h and V_l regions, which contributes to the stability of the molecule. Other synthetic constructs which can be used include CDR peptides. These are synthetic peptides comprising antigen-binding determinants. Peptide mimetics may also be used. These molecules are usually conformationally restricted organic rings which mimic the structure of a CDR loop and which include antigen-interactive side chains.

Synthetic constructs include chimaeric molecules. Thus, for example, humanised (or primatised) antibodies or derivatives thereof are within the scope of the present invention. An example of a humanised antibody is an antibody having human framework regions, but rodent hypervariable regions. Synthetic constructs also include molecules comprising an additional moiety that provides the molecule with some desirable property in addition to antigen binding. For example the moiety may be a label (e.g. a fluorescent or radioactive label). Alternatively, it may be a pharmaceutically active agent.

The antibodies or derivatives thereof of the present invention have a wide variety of uses in addition to their use in purification of polypeptides discussed above.

They can be used in therapy. For example they may be used to block undesirable ligand / receptor or receptor / receptor interactions.

They can be used in diagnosis. For example they may be used in RIAs or ELISAs in order to identify the presence or absence of the type 1 chemokine receptors that are within the scope of the present invention.

Nucleic Acid Molecules and Uses Thereof

The present invention also includes nucleic acid molecules within its scope.

Such nucleic acid molecules:

a) code for a polypeptide according to the present invention; or

b) are complementary to molecules as defined in a) above; or

c) hybridise to molecules as defined in a) or b) above.

These nucleic acid molecules and their uses will now be discussed in greater detail below:

The polypeptides of the present invention can be coded for by a large variety of nucleic acid molecules, taking into account the well-known degeneracy of the genetic code. All of these coding nucleic acid molecules are within the scope of the present invention. They may be administered to an individual and used to express polypeptides of the present invention. Thus they may be used for the same treatments as the polypeptides of the present invention. The nucleic acid molecules may be used directly, e.g. they may be injected into muscle (optionally after being first incorporated into a carrier, e.g. a lipid-based carrier, such as a liposome). Alternatively they may be inserted into vectors. Vectors for use in treatments include replication-deficient adenoviruses, retroviruses or adeno-associated viruses.

Vectors may be used in cloning. They may be introduced into host cells to enable the expression of polypeptides of the present invention using techniques known to the person

skilled in the art. Alternatively, cell free expression systems may be used. By using an appropriate expression system the polypeptides can be produced in a desired form. For example, the polypeptides can be produced by micro-organisms such as bacteria or yeast, by cultured insect cells (which may be baculovirus-infected), by mammalian cells (such as CHO cells) or by transgenic animals that, for instance, secrete the proteins in milk. Where glycosylation is desired, eukaryotic (desirably mammalian) expression systems are preferred.

Polypeptides comprising N-terminal methionine may be produced using certain expression systems, whilst in others the mature polypeptide will lack this residue. Polypeptides may initially be expressed to include signal sequences. Different signal sequences may be provided for different expression systems. Alternatively, signal sequences may be absent .

Techniques for cloning, expressing and purifying polypeptides are well known to the skilled person. Various such techniques are disclosed in standard text-books, such as in Sambrook *et al* [*Molecular Cloning* 2nd Edition, Cold Spring Harbor Laboratory Press (1989)]; in Old & Primrose [*Principles of Gene Manipulation* 5th Edition, Blackwell Scientific Publications (1994)]; and in Stryer [*Biochemistry* 4th Edition, W H Freeman and Company (1995)].

In addition to nucleic acid molecules coding for polypeptides of the present invention (referred to herein as "coding" nucleic acid molecules), the present invention also includes nucleic acid molecules complementary thereto. Thus, for example, both strands of a double stranded nucleic acid molecule are included within the scope of the present invention (whether or not they are associated with one another). Also included are mRNA molecules and complementary DNA molecules (e.g. cDNA molecules).

Nucleic acid molecules which can hybridise to one or more of the nucleic acid molecules discussed above are also within the scope of the present invention. Such nucleic acid molecules are referred to herein as "hybridising" nucleic acid molecules.

A hybridising nucleic acid molecule of the present invention may have a high degree of sequence identity along its length with a nucleic acid molecule within the scope of a) or b) above (e.g. at least 50%, at least 75% or at least 90% sequence identity).

As will be appreciated by the skilled person, the greater the degree of sequence identity that a given single stranded nucleic acid molecule has with another nucleic acid molecule, the greater the likelihood that it will hybridise to a nucleic acid molecule which is complementary to that other nucleic acid molecule under appropriate conditions.

Desirably hybridising molecules of the present invention are at least 10 nucleotides in length and preferably are at least 25 or at least 50 nucleotides in length.

Preferred hybridising molecules hybridise under stringent hybridisation conditions. One example of stringent hybridisation conditions is where attempted hybridisation is carried out at a temperature of from about 35°C to about 65°C using a salt solution which is about 0.9 molar. However, the skilled person will be able to vary such parameters as appropriate in order to take into account variables such as probe length, base composition, type of ions present, etc.

Most preferably, hybridising nucleic acid molecules of the present invention hybridise to a cDNA molecule having the sequence shown in Figure 3 or Figure 5; to an RNA equivalent thereof; or to a complementary sequence to any of the aforesaid molecules.

Hybridising nucleic acid molecules can be useful as probes or primers, for example. Probes can be used to purify and/or to identify nucleic acids. They may be used in

diagnosis. For example probes may be used to determine whether or not an individual has a receptor of the present invention by determining whether or not a complete gene coding for a functional receptor is present. Primers are useful in amplifying nucleic acids or parts thereof, e.g. by PCR techniques.

In addition to being used as probes or primers, hybridising nucleic acid molecules of the present invention can be used as antisense molecules to alter the expression of polypeptides of the present invention by binding to complementary nucleic acid molecules. (Generally this can be achieved by providing nucleic acid molecules that bind to RNA molecules that would normally be translated, thereby preventing translation due to the formation of duplexes.) This technique can be used in antisense therapy. Antisense molecules may be in the form of DNA or RNA molecules.

It is however important to note that nucleic acid molecules for use in the present invention include not only those with classical DNA or RNA structures, but also variants with modified (non-phosphodiester) backbones.. Two successful attempts to replace the entire backbone have been reported - the morpholino derivatives and the peptide nucleic acids (PNAs), which contain an N-(2-aminoethyl)glycine-based pseudopeptide backbone. (See Nielsen, P.E., *Annual Review of Biophysics & Biomolecular Structure*, **24** 167-83 (1995)). Nucleic acid variants with modified backbones can have increased stability relative to unmodified nucleic acids and are particularly useful where long-term hybridisation is desired (e.g. in antisense therapy).

Hybridising molecules may also be provided as ribozymes. Ribozymes can be used to regulate expression by binding to and cleaving RNA molecules that include particular target sequences.

From the foregoing discussion it will be appreciated that a large number of nucleic acids are within the scope of the present invention. Unless the context indicates otherwise,

nucleic acid molecules of the present invention may therefore have one or more of the following characteristics:

1) They may be DNA or RNA (including forms with non-naturally occurring bases and/or non-naturally occurring backbones e.g. PNAs).
2) They may be single or double stranded.
3) They may be provided in recombinant form i.e. covalently linked to a heterologous 5' and/or a 3' flanking sequence to provide a chimaeric molecule (e.g. a vector) which does not occur in nature.
4) They may be provided without 5' and/or 3' flanking sequences that normally occur in nature.
5) They may be provided in substantially pure form, e.g. by using probes to isolate cloned molecules having a desired target sequence or by using chemical synthesis techniques. (Thus they may be provided in a form which is substantially free from contaminating proteins and/or from other nucleic acids.);
6) They may be provided with introns (e.g. as a full-length gene) or without introns (e.g. as cDNA).

The present invention will now be described by way of example only with reference to the accompanying drawings; wherein :

Figure 1 shows an alignment of human and murine receptor amino acid sequences predicted from cDNA sequence information. Identical amino acid residues are boxed in black. The human and murine polypeptides are referred to as hGBRI-ILR and mGBRI-ILR respectively, since they are believed to be interleukin receptors (or at least substantial parts of such receptors).

Figure 2 shows an alignment of the hGBRI-ILR and mGBRI-ILR amino acid sequences shown in Figure 1 with members of the IL-6 type cytokine receptor family within the immunglobulin domain and haematopoietin receptor module. GCSF-R is granulocyte colony stimulating factor receptor and CNTF-R is ciliary neurotrophic factor.

Figure 3 shows the cDNA sequence obtained for hGBRI-ILR

Figure 4 shows the predicted amino acid sequence obtained from the cDNA sequence provided in Figure 3.

Figure 5 shows the cDNA sequence obtained for mGBRI-ILR

Figure 6 shows the predicted amino acid sequence obtained from the cDNA sequence provided in Figure 5.

Materials and Methods

Cloning of Human and Mouse cDNA for GBRI-ILR

The amino acid sequence N K L C F D D N K L W S D W S E A Q S I G K E Q N from murine the IL-13R was used to search the Genbank database with expressed sequence tags (ESTs) using TBLASTN, in order to identify the relevant EST. BLASTN searches of The Genbank database with this EST sequence allowed the identification of overlapping ESTs. The mouse cDNA clone 479043 was purchased from Research Genetics Inc. (Birmingham, AL). 5'-RACE was used to clone a further 310bp of the murine cDNA upstream of the 5' end of the cDNA clone using the Marathon cDNA amplification kit from Clonetech (Palo Alto, CA) on poly A+ RNA extracted from mouse lung following manufacturer's guidelines. The primer used in the PCR amplification (along with the AP-1 primer provided) was

5'-CGTACCACCTCAGCTTGTACTTG-3'. PCR products were cloned into the vector pCRII (Invitrogen, Leek, The Netherlands) and colonies screened by *colony hybridization* using the oligonucleotide probe 5'-AAGGATCTCACGTGCCGCTGGACACCGGGT-3'.

A portion of the human GBRI-ILR cDNA was amplified by PCR using cDNA derived from poly A+ RNA from human lung with the primers 5'-ACCGCCGAGGGCCTCTACTG-3' and 5'-TTGAGGGAGTAGTTGGTGTGGAGG-3'. Amplified products were cloned into the vector pCRII and colonies screened for the relevant insert by *colony hybridization* using the oligonucleotide probe 5'-TGAGCTCTCCCGTGTACTCAACGCCTCCAC-3'. This amplified DNA product was used as a probe to screen a human placental cDNA library in λgt10. The largest cDNA (1740bp) was recloned into pBluescript II SK-.

DNA and Protein Sequence Analysis

Sequences were obtained from cDNA clones by both strand automated sequencing, and were analyzed, along with all relevant ESTs, by the sequence analysis software Sequencher (Genecodes Corporation, Ann Arbor, MI). The signalP server (htpp://www.cbs.dtu.dk/signalp/cbssignalp.html) was used to identify the predicted cleavage site of the signal peptide for GBRI-ILR. DNA and amino acid sequence alignments as well as prediction of hydrophobic regions were analyzed with the Wisconsin package version 8.1 (Genetics Computer Group Inc., Madison,WI).

Detection of Human and Mouse mRNA Transcripts for GBRI-ILR

For detection of human GBRI-ILR mRNA transcripts by Northern blot analysis, the same 310bp cDNA used to screen the human placental library (see above) was used as

a probe. For detection of human gp130 mRNA, a partial cDNA was amplified with the primers 5'-CCGCGCAAGATGTTGACGTT-3' and 5'-CATTCGGACAGCTTGAACAG-3' and used as a probe. For detection of the human IL-6Rα, a partial cDNA was amplified with the primers 5'-CTGACCAGTCTGCCAGGAGACA-3' and 5'-GAGGACCCCACTCACAAACAAC-3' and used as a probe. The Human, Human II, Human Immune System, Human Endocrine and Human Fetal Multiple Tissue Northern Blots (Clonetech) were used to detect expression in human tissues. For human cells and cell lines, briefly, poly A+ RNA was isolated using the Oligotex Direct mRNA Mini Kit (Qiagen, Basel, Switzerland) following manufacturers guidelines. 0.5μg RNA was resolved on a formaldehyde gel and transferred to a Genescreen membrane (NEN Research Products, Boston, MA). All Northern blots were hybridised with the appropriate probes in ExpressHyb solution (Clonetech).

For detection of murine GBRI-ILR transcripts, either cDNA or cRNA probes were used. The cDNA probe was a product of PCR amplification using the primers 5'-CTAGGCTCAGCAAGATCTGATGTCC-3' and 5'-GCTCCAGATTCCCGCCTTTTTCGACC-3'. To generate cRNA probes, the PCR product generated with the primers 5'-CTGGCCCTGGCTAACCTTAATGG-3' and 5'-GCTCCAGATTCCCGCCTTTTTCGACC-3' was cloned into pBluescript II KS- (Stratagene) at the EcoRV restriction site. The plasmid was linearized with the restriction enzyme BlnI and cRNA probes labelled with ^{32}P by transcription with T3 RNA polymerase from the T3 promoter. The Mouse and Mouse Embryo Multiple Tissue Northern Blots (Clonetech), were hybridized with cDNA probe in ExpressHyb solution. For the other Northern blot analysis, mice were immunised with alum precipitated KLH sub-cutaneously and tissues removed at either day 0 or day 14 (with the exception of bone marrow, which was a pool of both). Total RNA was isolated with TRIzol reagent (Life Technologies AG, Basel, Switzerland) and polyA+ RNA was isolated using Oligotex beads (Qiagen). Northern blot analysis was performed

using the cRNA probe on 1.5μg polyA+ RNA as previously described by Gauchat, J-F. *et al*, (European Journal of Immunology, 1989 19:1079). For detection of the murine transcript by RT-PCR, 5μg of total RNA from the appropriate source was reverse transcribed using the first-strand cDNA synthesis kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) following manufacturer's guidelines. PCR was performed using the same primers used to amplify the murine DNA probe (see above). PCR products were transferred to a Genescreen hybridization membrane (NEN research products) and hybridised with 32P labelled oligonucleotide probe 5'-GCGGATCTGGTACTTGGTTTGAAAGAGGAA-3'.

Cloning and Distribution of the Receptor

Cloning of human GBRI-ILR

The Genbank database with expressed sequence tags (ESTs) was searched using TBLASTN with a 20 amino acid sequence surrounding the W-S-x-W-S motif of the mouse IL-13 receptor α1 as query. ESTs showing significant homology were then translated, and the open reading frames used to search the Swissprot database using BLASTP for homologous proteins. The amino acid sequence from the murine EST W66776 showed a high level of homology to members of the IL-6-type cytokine receptor family, as well as to the prolactin receptor. Using the sequence of W66776 to search the Genbank database allowed the identification of overlapping homologous sequences (of both murine and human origin) which in turn were run against the Genbank database to identify more overlapping ESTs (Table I). This allowed the assembly of overlapping nucleic acid sequences encoding the human and mouse putative receptor sequences.

To clone the human cDNA encoding GBRI-ILR, a 310bp PCR product was amplified from human lung cDNA using primers designed from the human ESTs. The PCR product was in turn used as a probe to screen a human placental cDNA library,

allowing the isolation of a full length clone of 1740 bp which included a 3' poly A tail. The human cDNA encoded a precursor protein of 422 amino acids with a putative signal peptide of 37 amino acids. In vitro translation revealed that the AUG codon coding the methionine at the start of the putative signal peptide was indeed used to initiate translation (data not shown).

Cloning of mouse GBRI-ILR

The Genbank database with expressed sequence tags (ESTs) was searched using TBLASTN with a 20 amino acid sequence surrounding the W-S-x-W-S motif of the mouse IL-13 receptor α1 as query. ESTs showing significant homology were then translated, and the open reading frames used to search the Swissprot database using BLASTP for homologous proteins. The amino acid sequence from the murine EST W66776 showed a high level of homology to members of the IL-6-type cytokine receptor family, as well as to the prolactin receptor. Using the sequence of W66776 to search the Genbank database allowed the identification of overlapping homologous sequences (of both murine and human origin) which in turn were run against the Genbank database to identify more overlapping ESTs (Table I). This allowed the assembly of overlapping nucleic acid sequences encoding the human and mouse putative receptor sequences.

The cDNA clone 479043, which gave rise to the mouse EST found furthest 5' in the sequence assembly was obtained from the IMAGE consortium and sequenced and was found to contain an insert of 1 Kb, including a 3' poly A tail. The rapid amplification of 5' cDNA ends (5'-RACE) on murine lung cDNA allowed the cloning of a further 308 bp upstream. The murine cDNA encoded a protein of 383 amino acids. The mouse cDNA sequence was incomplete at the 5' end as the first amino acid of the translated sequence aligned to amino acid 39 of the putative human receptor sequence, and no starting methionine or putative signal peptide could be identified.

Sequence Analysis

Sequence analysis of the human and murine cDNAs showed 85% sequence identity at the nucleic acid level and 96% identity at the amino acid level (Figure1). Amino acid identity between human and mouse gp130 is 77% and between the human and mouse prolactin receptors is 69%. As the level of identity between the human and murine putative receptors is significantly higher, this suggests a functionally important role for the GBRI-ILR. No putative transmembrane domain could be identified in either the human or mouse amino acid sequence, suggesting that the protein encoded by the cloned cDNAs are either secreted or GPI-anchored. As no hydrophobic region at the C terminus of the sequence, characteristic of GPI-anchored proteins such as the CNTF receptor, was identified, it is more likely that the cloned human and mouse cDNAs encode soluble receptors. There are numerous examples of soluble forms of receptors in the type I cytokine receptor family, being the product of either membrane shedding or alternative splicing. These soluble forms can exhibit either antagonistic effects in terms of ligand signalling such as those shown by soluble gp130 and the soluble IL-5 receptor α chain, or agonistic effects, such as those shown by the IL-6 receptor α chain, the CNTF receptor and the IL-11 receptor α chain.

Human and murine GBRI-ILR show close homology to members of the IL-6-type cytokine receptor family (Table II) as well as to the prolactin receptor when used as query to search the SwissProt database. Alignment of the human and mouse amino acid sequences to members of the IL-6-type cytokine receptor family showed regions of conserved homology within the two functionally important cytokine receptor-like domain, most notably at the highly conserved four cysteine residues and the W-S-x-W-S motif (Figure 2). The N-terminal domain of both sequences also appears to represent an Ig-like domain, most closely resembling the C2-set sequence.

One of the human EST's (H14009) showing homology to GBRI-ILR was a sequence derived from a genomic clone (D2-17). This clone was generated by exon

amplification of chromosomal DNA from human chromosome 19p12-13.1, allowing us to localise the human GBRI-ILR gene to this region. The gene for the erythropoeitin receptor, which shares significant homology with GBRI-ILR, is the only other member of the receptor family which has been shown to be localized to this arm of chromosome 19.

Distribution of Human and Mouse GBRI-ILR

mRNA expression was studied in human and mouse tissues by Northern blot analysis. The predominantly expressed form of the human mRNA migrated as a 1.7 Kb transcript, a size close to the one predicted from the clone obtained from the library screening. Another transcript of approximately 4.5Kb was seen in several tissues. This form could encode a membrane-anchored form of the receptor, analogous to the two transcripts detected for the IL-5 receptor α chain. Expression of the 1.7Kb transcript could be detected in several tissues, but was less ubiquitous than those of gp130 and IL-6Rα. Strongest expression of the human GBRI-ILR mRNA was detected in the spleen, thymus, lymph node, appendix, bone marrow, thyroid, adrenal cortex, stomach, heart, placenta and skeletal muscle. This distribution is compatible with a possible role for human GBRI-ILR in the immune system. Expression was also detected in several cell lines (Table III), such as the fibroblast cell line HEK 293, the monocyte cell line THP-1 (following PMA stimulation), JY lymphoblastoid cells, RPMI 8226 myeloma cells, the mast cell line HMC-1, bronchial epithelial cells HBE-140 and low level expression on HUVEC. In human fetal tissue, a strong expression was seen in the lung, but not in brain, kidney or liver.

In the adult mouse, expression of a 1.7Kb transcript was seen most strongly in the lung, but the transcript could also be detected at lower levels in skeletal muscle as well as heart and brain. Expression of the 1.7Kb transcript was also detected in the lymph node and thymus of immunized and non-immunized mice as well as in mouse bone marrow. In the embryo, the 1.7Kb transcript could first be detected at day 11 post

conception, with expression going through to days 15 and 17 post conception. This pattern of expression would appear to coincide with the emergence of the first detectable progenitors of the immune system, at day 10.5 post conception. Taken together, these data indicate a possible role for GBRI-ILR in the immune system and in embryonic development.

Overcoming cloning / expression difficulties

Several attempts to clone the full length cDNA for both human and mouse GBRI-ILR by PCR failed due to the lack of amplification of a significantly long product when attempting rapid amplification of 5' cDNA ends (5' RACE). This was later found to be due to the presence of a very GC rich region of the DNA at the 5' end of the cDNA, hampering the PCR reaction. This problem was overcome for hGBRI-ILR by screening a placental cDNA library with a cloned cDNA fragment obtained by PCR amplification using primers designed from the human ESTs.

Recombinant protein expression using the baculovirus expression system was also found to be less than efficient when using partial cDNA for hGBRI-ILR which was lacking the immunoglobulin domain. Higher levels of protein production were observed when cDNA encoding the complete N-terminal region of the protein was used.

Table I

Receptor	EST Accession no.	Tissue/Source of Origin
Mouse	AA014965	Placenta
	AA039053	Embryo
	AA049278	Embryo
	AA049280	Embryo
	AA270365	Embryo
	W17583	Embryo
	W66776	Embryo
Human	AA042914	Pregnant uterus
	AA043001	Pregnant uterus
	AA121532	Pregnant uterus
	AA127694	Pregnant uterus
	AA377893	Synovial Sarcoma
	AA406406	Melanocyte/Fetal Heart/uterus
	H14009	Chromosome 19
	N78873	Fetal Lung
	R87407	Brain
	W37175	Fetal Lung
	W46603	Fibroblast
	W46604	Fibroblast

Table II

Cloned Receptor	Alignment With	Identical Residues	Similar Residues
Human	GP130	87/306	120/306
	GCSF Receptor	85/306	112/306
	CNTF Receptor	79/306	102/306
	IL6 Receptor alpha chain	71/306	104/306
Mouse	GP130	89/304	125/304
	GCSF	81/304	115/304

	Receptor IL6Receptor alpha chain	71/304	107/304

Table III

Cell/Cell Line	hGB RI-ILR	Cell/Cell Line	hGB RI-ILR
TT7	-	HL60	-
JURKAT	-	HMC-1	++
JURKAT (+PMA/Ionomycin)	-	HBE-140	-/+
JY	+	HBE-140 (IL-1β)	+
JY (+IL-4)	++	HBE-140 (TNFα)	+
RPMI 8866	-	HEK 293	++
RPMI 8226	+	HUVEC	-/+
THP-1	-	HUVEC (IL-1β/TNFα 2hr)	-/+
THP-1 (+PMA)	+	HUVEC (IL-1β/TNFα8hr)	-/+

Example of preparation of recombinant soluble GBRI-ILR

Soluble human GBRI-ILR cDNA, truncated at amino acid 378, and coding for the 6 histidine and 179 recognition tags at the 3' end was cloned into pFASTBAC1. Recombinant virus was produced using the Life Technologies BAC-TO-BAC kit and used to infect SF9 cells expanded in SF900II medium. Protein secreted into the medium was purified using a NI-NTA resin column (which binds the 6 histidines). Purified protein was detected by western blot analysis using a monoclonal antibody recognising the 179 tag (CLEPYTACD).

Example of the generation of hybridomas producing anti-GBRI-ILR monoclonal antibodies

A Balb/c mouse was immunized on day 0, 7 and 28 subcutaneously in the limbs and behind the neck with 100 μg of protein per injection. Three days after the final injection, the draining lymph nodes were obtained and the tissue digested using a collagenase and DNAse cocktail according to the procedure reported elsewhere (Kosco-Vilbois M.H.,Isolation and Enrichment of Follicular Dendritic Cells from Murine Lymphoid Tissue, *Immunology Methods Manual*, **Vol 3**, 1997). The resulting cell suspension was resuspended at 10^6 cells per ml and fused with Sp2 myeloma cells using standard "Kohler and Milstein" protocols. The hybridomas were then selected in HAT medium and 7-10 days after fusion, the supernatants harvested for screening.

To screen the hybridoma supernatants, 96 wells plates (Falcon 3912; Becton Dickinson Labware Europe, Meylan, France) were coated overnight at 4°C with 1 μg/ml soluble GBRI-ILR purified from infected Sf9 cell in carbonate buffer pH 9.6. Plates were then washed, blocked with PBS containing 1% BSA and incubated for two hours with 200 μl hybridoma supernatant and washed. GBRI-ILR specific mAbs were revealed using horseradish peroxidase labelled goat anti-mouse IgG (Southern Biotechnology Associates, Inc.) Positive supernatants were retested with plastic immobilized GBRI-ILR. Specificity was checked using an ELISA set up with IL-13Rα1 at 1 μg/ml. The specific positive supernatants were further screened by FACS using HEK-293 cells transfected with a cDNA encoding a fusion protein between GBRI-ILR up to amino acid 354 and the IL-13 Rα1 transmembrane and cytoplasmic regions, IL-13Rα1 cDNA or an empty plasmid (the plasmid used for expression was pEBS). Hybridomas that demonstrated the strongest fluorescence signal on GBRI-ILR transfectants and also did not bind to control proteins or transfectants were retained for further use.

Claims

1. A polypeptide, which:

a) has the amino acid sequence of amino acids 38 to 422 shown in Figure 1 for hGBRI-ILR, or which has the amino acid sequence shown in Figure 1 for mGBRI-ILR;

b) has one or more amino acid deletions, insertions or substitutions relative to a polypeptide as defined in a) above, but has at least 40% amino acid sequence identity therewith; or

c) is a fragment of a polypeptide as defined in a) or b) above, which is at least 10 amino acids long.

2. A polypeptide according to claim 1, which has a haematopoietin receptor module.

3. A polypeptide according to claim 1 or claim 2, which comprises the amino acid sequence of amino acids 38 to 422 shown in Figure 1 for hGBRI-ILR, or the amino acid sequence shown in Figure 1 for mGBRI-ILR.

4. A polypeptide according to any preceding claim, in glycosylated form.

5. A polypeptide according to any preceding claim, in soluble form.

6. A pharmaceutically acceptable composition comprising a polypeptide according to any preceding claim.

7. A polypeptide according to any of claims 1 to 5 or a pharmaceutically acceptable composition according to claim 6, for use in medicine.

8. The use of a polypeptide according to any of claims 1 to 5 in the manufacture of a medicament for treating cancer, an immune disorder, obesity or a developmental disorder.

9. The use of a polypeptide according to any of claims 1 to 5 in the manufacture of a medicament for treating AIDS, septic shock, embryonic developmental disorders or lung inflammation.

10. The use of a polypeptide according to any of claims 1 to 5 in screening.

11. The use according to claim 10 in screening for a cytokine that binds to a type 1 cytokine receptor.

12. The use according to claim 10 in screening for an agonist or antagonist of a cytokine that binds to a type 1 cytokine receptor.

13. A cytokine that binds to a type 1 cytokine receptor, or an agonist or an antagonist thereof identifiable by or identified by screening as described in any of claims 10 to 12.

14. A cytokine, an agonist or an antagonist according to claim 13, for use in medicine.

15. The use of a cytokine, an agonist or an antagonist according to claim 12 in the manufacture of a medicament for treating cancer, an immune disorder, obesity or a developmental disorder.

16. The use according to claim 10 in screening for an agonist or antagonist of a cytokine that binds to a type 1 cytokine receptor in the manufacture of a

medicament for treating AIDS, septic shock, embryonic developmental disorders or lung inflammation.

17. The use of a polypeptide according to any of claims 1 to 5 in raising or selecting antibodies.

18. An antibody or a derivative thereof which binds to a polypeptide according to any of claims 1 to 5.

19. A pharmaceutically acceptable composition comprising an antibody or a derivative thereof according to claim 18.

20. An antibody or a derivative thereof according to claim 18 or a pharmaceutically acceptable composition according to claim 19, for use in medicine.

21. The use of an antibody or a derivative thereof according to claim 20 in the preparation of a medicament for treating cancer, an immune disorder, obesity or a developmental disorder.

22. The use of an antibody or a derivative thereof according to claim 20 in the manufacture of a medicament for treating AIDS, septic shock, embryonic developmental disorders or lung inflammation.

23. A nucleic acid molecule, which:

a) codes for a polypeptide according to any of claims claim 1 to 5,

b) is complementary to a molecule as defined in a) above, or

c) hybridises to a molecule as defined in a) or b) above.

24. A vector comprising a nucleic acid molecule according to claim 23.

25. A host comprising a nucleic acid molecule according to claim 23 or a vector according to claim 24.

26. A method for obtaining a polypeptide according to any of claims 1 to 5, comprising incubating a host according to claim 25 under conditions causing expression of said polypeptide and then purifying said polypeptide.

27. A nucleic acid molecule, vector or host according to any of claims 23, 24 or 25 respectively, for use in medicine.

28. The use of a nucleic acid molecule, vector or host according to any of 23, 24 or 25 respectively in the preparation of a medicament for antisense therapy.

29. The use of nucleic acid molecule according to claim 23 as a probe or as a primer.

30. The invention as substantially hereinbefore described.

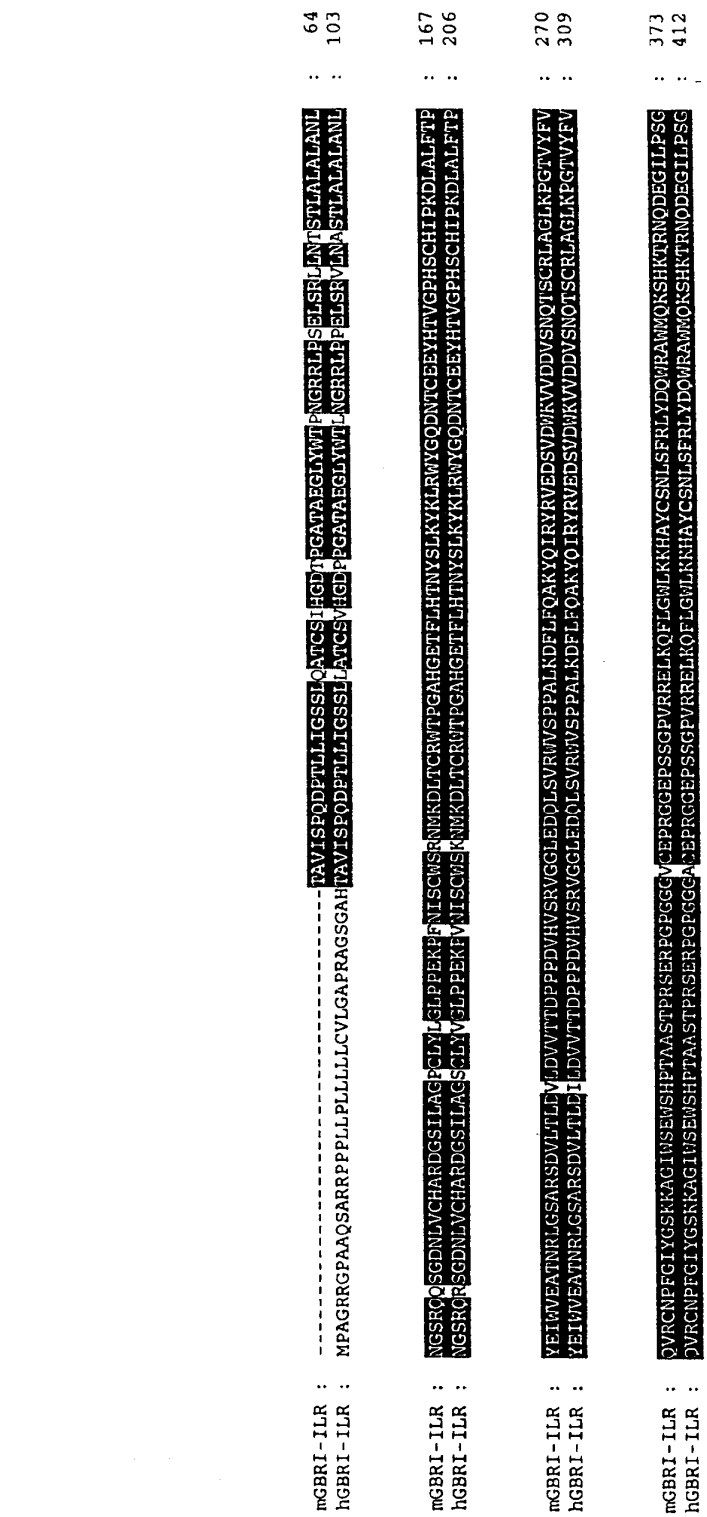

mGBRI-ILR : ----------TAVISPQDPTLLIGSSLQATCSIHGDTPGATAEGLYWTPNGRRLPSELSRLLNTSTLALALANL : 64
hGBRI-ILR : MPAGRRGPAAQSARRPPPLLPLLLLLCVLGAPRAGSGAHTAVISPQDPTLLIGSSLLATCSVHGDFPGATAEGLYWTLNGRRLPPELSRVLNASTLALALANL : 103
mGBRI-ILR : NGSRQQSGDNLVCHARDGSILAGPCLYLGLPPEKPFNISCWSRNMKDLTCRWTPGAHGETFLHTNYSLKYKLRWYGQDNTCEEYHTVGPHSCHIPKDLALFTP : 167
hGBRI-ILR : NGSRQRSGDNLVCHARDGSILAGSCLYVGLPPEKPVNISCWSKNMKDLTCRWTPGAHGETFLHTNYSLKYKLRWYGQDNTCEEYHTVGPHSCHIPKDLALFTP : 206
mGBRI-ILR : YEIWVEATNRLGSARSDVLTLDVDVTTDPPPDVHVSRVGGLEDQLSVRWVSPPALKDFLFQAKYQIRYRVEDSVDWKVVDDVSNQTSCRLAGLKPGTVYFV : 270
hGBRI-ILR : YEIWVEATNRLGSARSDVLTLDILDVVTTDPPPDVHVSRVGGLEDQLSVRWVSPPALKDFLFQAKYQIRYRVEDSVDWKVVDDVSNQTSCRLAGLKPGTVYFV : 309
mGBRI-ILR : QVRCNPFGIYGSKKAGIWSEWSHPTAASTPRSERPGPGGCVCEPRGGEPSSGPVRRELKQFLGWLKKHAYCSNLSFRLYDQWRAWMQKSHKTRNQDEGILPSG : 373
hGBRI-ILR : QVRCNPFGIYGSKKAGIWSEWSHPTAASTPRSERPGPGGCACEPRGGEPSSGPVRRELKQFLGWLKKHAYCSNLSFRLYDQWRAWMQKSHKTRNQDEGILPSG : 412
mGBRI-ILR : RRGAARGPAG : 383
hGBRI-ILR : RRGTARGPAR : 422

FIG. 1

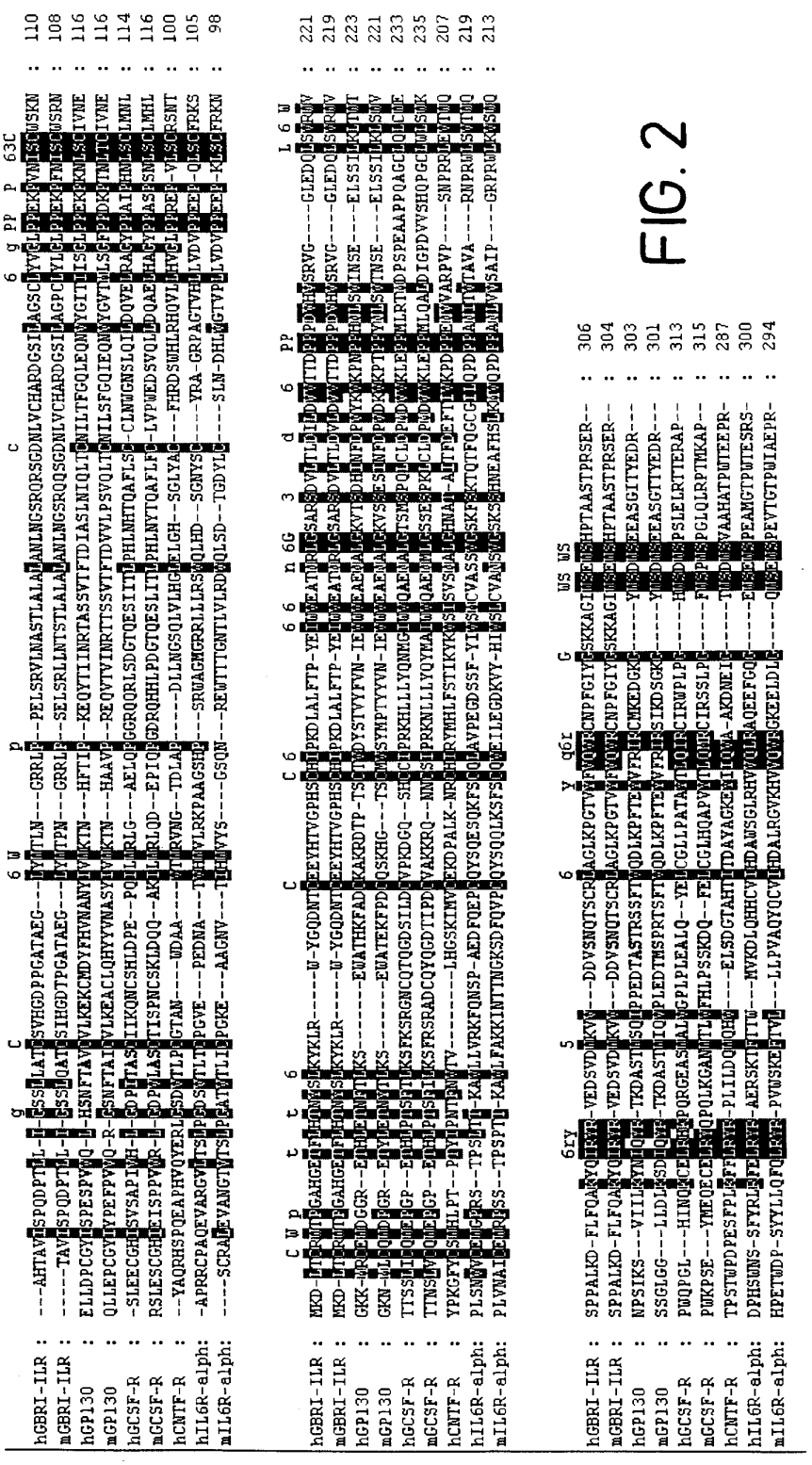


FIG. 2

FIG. 3

```
   1  CGCCCAGCGA CGTGCGGGCG GCCTGGCCCG CGCCCTCCCG CGCCCGGCCT
  51  GCGTCCCGCG CCCTGCGCCA CCGCCGCCGA GCCGCAGCCC GCCGCGCGCC
 101  CCCGGCAGCG CCGGCCCCAT GCCCGCCGGC CGCCGGGGCC CCGCCGCCCA
 151  ATCCGCGCGG CGGCCGCCGC CGTTGCTGCC CCTGCTGCTG CTGCTCTGCG
 201  TCCTCGGGGC GCCGCGAGCC GGATCAGGAG CCCACACAGC TGTGATCAGT
 251  CCCCAGGATC CCACGCTTCT CATCGGCTCC TCCCTGCTGG CCACCTGCTC
 301  AGTGCACGGA GACCCACCAG GAGCCACCGC CGAGGGCCTC TACTGGACCC
 351  TCAACGGGCG CCGCCTGCCC CCTGAGCTCT CCCGTGTACT CAACGCCTCC
 401  ACCTTGGCTC TGGCCCTGGC CAACCTCAAT GGGTCCAGGC AGCGGTCGGG
 451  GGACAACCTC GTGTGCCACG CCCGTGACGG CAGCATCCTG GCTGGCTCCT
 501  GCCTCTATGT TGGCCTGCCC CCAGAGAAAC CCGTCAACAT CAGCTGCTGG
 551  TCCAAGAACA TGAAGGACTT GACCTGCCGC TGGACGCCAG GGGCCCACGG
 601  GGAGACCTTC CTCCACACCA ACTACTCCCT CAAGTACAAG CTTAGGTGGT
 651  ATGGCCAGGA CAACACATGT GAGGAGTACC ACACAGTGGG GCCCCACTCC
 701  TGCCACATCC CCAAGGACCT GGCTCTCTTT ACGCCCTATG AGATCTGGGT
 751  GGAGGCCACC AACCGCCTGG GCTCTGCCCG CTCCGATGTA CTCACGCTGG
 801  ATATCCTGGA TGTGGTGACC ACGGACCCCC CGCCCGACGT GCACGTGAGC
 851  CGCGTCGGGG GCCTGGAGGA CCAGCTGAGC GTGCGCTGGG TGTCGCCACC
 901  CGCCCTCAAG GATTTCCTCT TTCAAGCCAA ATACCAGATC CGCTACCGAG
 951  TGGAGGACAG TGTGGACTGG AAGGTGGTGG ACGATGTGAG CAACCAGACC
1001  TCCTGCCGCC TGGCCGGCCT GAAACCCGGC ACCGTGTACT TCGTGCAAGT
1051  GCGCTGCAAC CCCTTTGGCA TCTATGGCTC CAAGAAAGCC GGGATCTGGA
1101  GTGAGTGGAG CCACCCCACA GCCGCCTCCA CTCCCCGCAG TGAGCGCCCG
1151  GGCCCGGGCG GCGGGGCGTG CGAACCGCGG GGCGGAGAGC CGAGCTCGGG
```

```
1201  GCCGGTGCGG CGCGAGCTCA AGCAGTTCCT GGGCTGGCTC AAGAAGCACG
1251  CGTACTGCTC CAACCTCAGC TTCCGCCTCT ACGACCAGTG GCGAGCCTGG
1301  ATGCAGAAGT CGCACAAGAC CCGCAACCAG GACGAGGGGA TCCTGCCCTC
1351  GGGCAGACGG GGCACGGCGA GAGGTCCTGC CAGATAAGCT GTAGGGGCTC
1401  AGGCCACCCT CCCTGCCACG TGGAGACGCA GAGGCCGAAC CCAAACTGGG
1451  GCCACCTCTG TACCCTCACT TCAGGGCACC TGAGCCACCC TCAGCAGGAG
1501  CTGGGGTGGC CCCTGAGCTC CAACGGCCAT AACAGCTCTG ACTCCCACGT
1551  GAGGCCACCT TTGGGTGCAC CCCAGTGGGT GTGTGTGTGT GTGTGAGGGT
1601  TGGTTGAGTT GCCTAGAACC CCTGCCAGGG CTGGGGGTGA GAAGGGGAGT
1651  CATTACTCCC CATTACCTAG GGCCCCTCCA AAAGAGTCCT TTTAAATAAA
1701  TGAGCTATTT AGGTGC
```

FIG. 3 CONT'D

```
   1  MPAGRRGPAA QSARRPPPLL PLLLLLCVLG APRAGSGAHT AVISPQDPTL
  51  LIGSSLLATC SVHGDPPGAT AEGLYWTLNG RRLPPELSRV LNASTLALAL
 101  ANLNGSRQRS GDNLVCHARD GSILAGSCLY VGLPPEKPVN ISCWSKNMKD
 151  LTCRWTPGAH GETFLHTNYS LKYKLRWYGQ DNTCEEYHTV GPHSCHIPKD
 201  LALFTPYEIW VEATNRLGSA RSDVLTLDIL DVVTTDPPPD VHVSRVGGLE
 251  DQLSVRWVSP PALKDFLFQA KYQIRYRVED SVDWKVVDDV SNQTSCRLAG
 301  LKPGTVYFVQ VRCNPFGIYG SKKAGIWSEW SHPTAASTPR SERPGPGGGA
 351  CEPRGGEPSS GPVRRELKQF LGWLKKHAYC SNLSFRLYDQ WRAWMQKSHK
 401  TRNQDEGILP SGRRGTARGP AR
```

FIG. 4

FIG. 5

```
   1  CACAGCTGTA ATCAGCCCCC AGGACCCCAC CCTTCTCATC GGCTCCTCCC
  51  TGCAAGCTAC CTGCTCTATA CATGGAGACA CACCTGGGGC CACCGCTGAG
 101  GGGCTCTACT GGACCCTCAA TGGTCGCCGC CTGCCCTCTG AGCTGTCCCG
 151  CCTCCTTAAC ACCTCCACCC TGGCCCTGGC CCTGGCTAAC CTTAATGGGT
 201  CCAGGCAGCA GTCAGGAGAC AATCTGGTGT GTCACGCCCG AGACGGCAGC
 251  ATTCTGGCTG GCTCCTGCCT CTATGTTGGC TTGCCCCCTG AGAAGCCCTT
 301  TAACATCAGC TGCTGGTCCC GGAACATGAA GGATCTCACG TGCCGCTGGA
 351  CACCGGGTGC ACACGGGGAG ACATTCTTAC ATACCAACTA CTCCCTCAAG
 401  TACAAGCTGA GGTGGTACGG TCAGGATAAC ACATGTGAGG AGTACCACAC
 451  TGTGGGCCCT CACTCATGCC ATATCCCCAA GGACCTGGCC CTCTTCACTC
 501  CCTATGAGAT CTGGGTGGAA GCCACCAATC GCCTAGGCTC AGCAAGATCT
 551  GATGTCCTCA CACTGGATGT CCTGGACGTG GTGACCACGG ACCCCCCACC
 601  CGACGTGCAC GTGAGCCGCG TTGGGGGCCT GGAGGACCAG CTGAGTGTGC
 651  GCTGGGTCTC ACCACCAGCT CTCAAGGATT TCCTCTTCCA AGCCAAGTAC
 701  CAGATCCGCT ACCGCGTGGA GGACAGCGTG GACTGGAAGG TGGTGGATGA
 751  CGTCAGCAAC CAGACCTCCT GCCGTCTCGC GGGCCTGAAG CCCGGCACCG
 801  TTTACTTCGT CCAAGTGCGT TGTAACCCAT TCGGGATCTA TGGGTCGAAA
 851  AAGGCGGGAA TCTGGAGCGA GTGGAGCCAC CCCACCGCTG CCTCCACCCC
 901  TCGAAGTGAG CGCCCGGGCC CGGGCGGCGG GGTGTGCGAG CCGCGGGGCG
 951  GCGAGCCCAG CTCGGGCCCG GTGCGGCGCG AGCTCAAGCA GTTCCTCGGC
1001  TGGCTCAAGA AGCACGCATA CTGCTCGAAC CTTAGTTTCC GCCTGTACGA
1051  CCAGTGGCGT GCTTGGATGC AGAAGTCACA CAAGACCCGA AACCAGGACG
1101  AGGGGATCCT GCCCTCGGGC AGACGGGGTG CGGCGAGAGG TCCTGCCGGC
1151  TAAACTCTAA GGATAGGCCA TCCTCCTGCT GGGTCAGACC TGGAGGCTCA
1201  CCTGAATTGG AGCCCCTCTG TACCATCTGG GCAACAAAGA AACCTACCAG
1251  AGGCTGGGGC ACAATGAGCT CCCACAACCA CAGCTTTGGT CCACATGATG
```

```
1301  GTCACACTTG GATATACCCC AGTGTGGGTA GGGTTGGGGT ATTGCAGGGC
1351  CTCCCAAGAG TCTCTTTAAA TAAATAAAGG AGTTGTTCAG TCCCGAGAAA
1401  AAAAAAAAAA AAAAAAATTT CCGCGGCCGC
```

FIG. 5CONT'D

```
   1  TAVISPQDPT LLIGSSLQAT CSIHGDTPGA TAEGLYWTLN GRRLPSELSR
  51  LLNTSTLALA LANLNGSRQQ SGDNLVCHAR DGSILAGSCL YVGLPPEKPF
 101  NISCWSRNMK DLTCRWTPGA HGETFLHTNY SLKYKLRWYG QDNTCEEYHT
 151  VGPHSCHIPK DLALFTPYEI WVEATNRLGS ARSDVLTLDV LDVVTTDPPP
 201  DVHVSRVGGL EDQLSVRWVS PPALKDFLFQ AKYQIRYRVE DSVDWKVVDD
 251  VSNQTSCRLA GLKPGTVYFV QVRCNPFGIY GSKKAGIWSE WSHPTAASTP
 301  RSERPGPGGG VCEPRGGEPS SGPVRRELKQ FLGWLKKHAY CSNLSFRLYD
 351  QWRAWMQKSH KTRNQDEGIL PSGRRGAARG PAG*TLRIGH PPAGSDLEAH
 401  LNWSPSVPSG QQRNLPEAGA Q
```

FIG. 6

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 June 2005 (30.06.2005)

PCT

(10) International Publication Number
WO 2005/058956 A1

(51) International Patent Classification[7]: C07K 14/715, C12N 15/12

(21) International Application Number:
PCT/AU2004/001762

(22) International Filing Date:
16 December 2004 (16.12.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2003906979 16 December 2003 (16.12.2003) AU

(71) Applicant *(for all designated States except US)*: **COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION** [AU/AU]; Limestone Avenue, Campbell, Australian Capital Territory 2601 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **VARGHESE, Joseph, Noozhumutry** [AU/AU]; 179 Nicholson Street, BRUNSWICK EAST, VIC 3057 (AU). **HUDSON, Peter, John** [AU/AU]; 36 Fuchsia Street, BLACKBURN, VIC 3130 (AU). **POWERS, Barbara, Elaine** [AU/AU]; 36 Fuchsia Street, Blackburn, VIC 3130 (AU).

(74) Agent: **F B RICE & CO**; 139-141 Landsdowne Street, Carlton, VIC 3053 (AU).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2005/058956 A1

(54) Title: CYTOKINE BINDING DOMAINS

(57) Abstract: The present invention relates a method of generating modified binding moieties comprising a cytokine binding domain consisting of a first FnIII-like domain and a second FnIII-like domain in which at least one amino acid residue within the cytokine binding domain in modified such that the binding characteristics of the cytokine binding domain are altered, and/or, the solubility and/or stability of the binding moiety is improved. The invention also relates to binding moieties and to the use of such binding moieties as affinity reagents, diagnostic reagents, therapeutic agents and protein scaffolds.

Cytokine binding domains

Field of the invention

The present invention relates to binding moieties derived from cytokine binding domains (CBDs) and their use as affinity reagents, diagnostic reagents, therapeutic agents and as protein scaffolds.

Background to the invention

Antibodies are the paradigm of specific high-affinity binding reagents and provide an antigen binding site by interaction of variable heavy (V_H) and variable light (V_L) immunoglobulin domains. The binding interface is formed by six surface polypeptide loops, termed complementarity determining regions (CDRs), three from each variable domain, which are highly variable and combined provide a sufficiently large surface area for interaction with antigen. Specific binding reagents can be formed by association of only the V_H and V_L domains into an Fv module. Bacterial expression is enhanced by joining the V-domains with a linker polypeptide into a single-chain scFv molecule.

WO 00/34784 and WO 01/64942 (Phylos Inc.) disclose antibody mimics comprising a fibronectin or fibronectin-like protein scaffold in which a fibronectin type III domain having at least one randomised loop is present. WO 02/32925 (Phylos Inc.) relates to non-antibody derivative proteins comprising a domain having an immunoglobulin-like fold in which the protein has a mutated amino acid sequence such that its binds to a compound with greater affinity than the unmutated protein.

Koide *et al.* (WO 98/56915 and J. Mol. Biol., (1998), 284, 1141-1151) describe the design and construction of a fibronectin type III domain scaffold and the use of the scaffold to produce a phage display library with mutation in two loops to screen for higher affinity ligand binding.

WO 01/90192 (Imclone Systems Inc) describes a bispecific two-chain immunoglobulin construct, a two domain protein which is optimized in its avidity for antigen but still acts as a natural antibody.

WO 02/48189 (Borean Pharma AS) describes a scaffold based on the family of C-type lectin-like domains, which has a carbohydrate recognition domain having a loop region that can be mutated so as to provide a new class of libraries.

WO 00/47620 (Medvet Science Pty Ltd *et al.*) discloses a cytokine-binding domain that consists of a β-chain or analogous structure of a cytokine receptor.

WO 02/44197 (Fish) describes cytokine receptor binding peptide constructs in which the cytokine receptor binding domain is incorporated into a scaffold such that the

scaffold maintains the binding domain configuration suitable for binding to the cytokine receptor.

Summary of the invention

The present invention relates to binding moieties which employ a CBD-like scaffold structure consisting of two FnIII-like domains as schematically depicted in Figure 1A. Solvent exposed loops on the two FnIII-like domains are in linear association and define a binding region which is capable of binding to a target molecule through association with loops from both domains. The invention also relates to a method for producing novel scaffold structures based on the use of cytokine-binding domains (CBDs) as well as the novel scaffold structures produced thereby.

Accordingly, the invention provides to a method of producing a binding moiety comprising modifying an extracellular cytokine binding domain consisting of a first FnIII-like domain and a second FnIII-like domain such that at least one property of the cytokine binding domain is altered, to produce a binding moiety.

Furthermore, the invention provides a modified binding moiety produced according to the above method of the invention.

The present invention also provides novel binding moieties based on a cytokine binding domain scaffold structure.

Accordingly, the invention also provides a binding moiety comprising an extracellular cytokine binding domain consisting of a first FnIII-like domain and a second FnIII-like domain, wherein the CBD comprises a modification which alters a property of the CBD.

CBDs consist of two linked fibronectin type III (FnIII) domains (each an Ig-like fold) (Leahy DJ *et al.,* 1992, Science 258: 987-991). These CBDs are known to bind their target molecules primarily at the juncture of the two FnIII-like domains (the cytokine hinging region), engaging their target molecules by loops on the outer elbow of the two domains of the CBD. These loops are similar to the CDR (complementarity determining region) loops found on the antigen-binding surface of antibody variable domains. However, the association between loops from the two domains in a CBD exhibits important differences to antibody CDR loop association. In antibody variable domains, the loops from the heavy chain associate in parallel with those of the light chain. In contrast, the cytokine binding loops of cytokine binding regions form a linear association (see Figure 1). A comparison between the CBDs of a number of know tertiary structures reveal common structural features indicating that these domains form an ideal framework for designing and generating novel binding moieties. Such binding moieties will have a variety of uses and applications including, as diagnostic and therapeutic agents/reagents,

being directed to particular molecular targets, and in particular those targets associated with clinical disease.

The prior art typically describes scaffold structures based on single binding domains. In particular, previous work on scaffolds utilising FnIII-like domains has concentrated on the use of single FnIII-like domain frameworks. In contrast, the scaffolds of the invention are based on the use of CBDs having two FnIII-like domains, in which a target molecule can be bound through association with both domains, and more particularly through interaction with loops forming the cytokine binding region of the CBD.

The scaffolds of the invention provide significant advantages over the prior art scaffolds. The use of a two-domain binding moiety results in a larger surface binding area or "footprint" for binding with a target molecule. This creates the potential for binding with higher affinity and/or to a greater variety of target shapes and sizes. In particular, the use of a two-domain, linearly associated framework creates the potential for these moieties to bind to molecules that are refractory to conventional antibodies.

The binding moieties of the invention may be linked to other molecules, for example by covalent or non-covalent means. Accordingly, the invention provides a binding moiety according to the invention linked to one or more other molecules.

Furthermore, the invention provides a multivalent or multispecific reagent comprising two or more binding moieties according to the invention.

The invention also provides a polynucleotide encoding a binding moiety, multivalent reagent or multispecific reagent according to the invention.

The invention also provides a vector comprising a polynucleotide according to the invention.

The invention also provides a host cell comprising a vector according to the invention.

In addition, the invention provides a pharmaceutical composition comprising a binding moiety, multivalent reagent or multispecific reagent according to the invention and a pharmaceutically acceptable carrier, diluent, adjuvant and/or immunostimulant.

The invention also provides a method of treating a pathological condition in a subject, which method comprises administering to the subject binding moiety, multivalent reagent or multispecific reagent according to the invention.

The invention also provides a method of selecting a binding moiety with an affinity for a target molecule which comprises

(i) providing a plurality of polynucleotides encoding binding moieties comprising a CBD, which polynucleotides comprise one or more modifications in the CBD;

(ii) expressing the binding moieties encoded by the polynucleotides; and

(iii) selecting one or more binding moieties having an affinity for the target molecule.

The invention also provides a polynucleotide library comprising a plurality of polynucleotides encoding binding moieties comprising a cytokine binding domain, which polynucleotides comprise one or more modifications in the cytokine biding domain.

The invention also provides expression vectors useful in the generation of binding moieties according to the invention. Accordingly, the invention provides an expression vector comprising:

a) a first nucleic acid sequence encoding a CBD;

b) an insertion site in a region between the ends of the first nucleic acid sequence, the insertion site comprising a nucleotide sequence unique to said expression vector which is cleaved by a restriction endonuclease and which allows a second nucleic acid sequence encoding an amino acid sequence to be inserted into the first nucleic acid to encode a modified CBD; and

c) a regulatory control sequence operably linked to said first nucleic acid sequence which directs expression of the first nucleic acid sequence.

Preferably, the region encodes a solvent exposed region, preferably a loop.

The invention also provides an expression vector comprising:

a) a first nucleic acid sequence encoding a CBD, said sequence comprising a deletion in a region between the ends of the first nucleic acid sequence;

b) an insertion site in place of the deleted sequence which site allows a second nucleic acid sequence encoding an amino acid sequence to be inserted into the first nucleic acid to encode a modified CBD.

c) a regulatory control sequence operably linked to said first nucleic acid sequence which directs expression of the first nucleic acid sequence.

Preferably, the region encodes a solvent exposed region, preferably a loop.

The invention also provides an expression vector comprising:

a) a first nucleic acid sequence encoding a CBD;

b) a number of insertion sites in regions between the ends of the first nucleic acid sequence, each insertion site comprising a nucleotide sequence unique to said expression vector which is cleaved by a restriction endonuclease and which allows a nucleic acid sequence encoding an amino acid sequence to be inserted into the first nucleic acid to encode a modified CBD.

Preferably, one or more regions, preferably each region, encodes a solvent exposed region, preferably a loop.

The invention also provides a nucleic acid sequence encoding a peptide display scaffold comprising:

a) a first scaffold sequence encoding a CBD; and

b) a second sequence encoding a peptide and inserted at a site located in a region of said first scaffold sequence encoding a cytokine binding loop.

The invention also provides an expression vector comprising a nucleic acid sequence according to the invention described immediately above, as well as a CDB display library comprising a plurality of said expression vectors.

The invention also provides a polypeptide encoded by the nucleic acid sequence according to the invention described above, as well as a protein multimer comprising at least two of said polypeptides.

The invention also provides a method of identifying a modified CBD which binds to a target molecule of interest, which method comprises:

(i) providing a CBD display library of the invention;

(ii) expressing the polypeptides encoded by the polynucleotides; and

(iii) selecting one or more polypeptides that bind to the target molecule.

The various features and embodiments of the present invention, referred to in individual sections above apply, as appropriate, to other sections, mutatis mutandis. Consequently features specified in one section may be combined with features specified in other sections, as appropriate.

Description of the Figures

Figure 1. (a) A coil representation of the backbone of a CBD, illustrated by the CBD of the IL-6 receptor (IL-6R) (D2 representing the N-terminal domain and D3 representing the C-terminal domain). The loops marked L1 to L4 and L5 to L7 respectively, represent the loops from the N-terminal and C-terminal domains of the CBD that can engage a target macromolecule: (b) The view with the molecule rotated 90° with the seven loops facing up: (c) A coil representation of the backbone of the variable domains of the heavy and light chains of the Fv domain of an immunoglobulin, illustrated by the NC10 anti-neuraminidase Fv domain, showing the Fv domain's respective antigen binding CDRs as loops L1, L2, L3, H1, H2 and. (d) the view with the molecule rotated 90° with the loops facing up.

Both molecules are draw to scale, and it can been seen that while the Fv antigen binding site is approximately isotropic in distribution, the CBD loops are long and narrow, offering a different type of surface topology when compared to the potential binding site of antibody molecules.

Figure 1A : A schematic representation of a binding moiety according to the present invention. The CBD-like scaffold structure consists of a first and a second FnIII-like domain (indicated as $FnIII^1$ and $FnIII^2$). Solvent exposed loops present on each FnIII-like domains define a binding region capable of association with a target molecule. The binding region is essentially defined by solvent exposed loops presented by both domains.

Figure 2. (a) A ribbon diagram of the CBD of IL-6R, showing the β-sheet arrangement of the two FnIII domains, and the cytokine binding loops L1 to L7. (b) the same as in (a) but rotated 90° with the loops facing up.

Figure 3. (a) The amino acid sequence of IL-6R extracellular domain, showing the CBD comprising domain D2 (residues 92 to 195) and domain D3 (residues 196 to 297). The position of β-sheet structures are indicated by #. The position of loops in the cytokine binding region are shown by * and marked L1 to L7. The Pro94, Pro95, Cys102, Cys103, Trp115, Cys146, Cys157, Pro199, Pro200, Trp219, Arg274, Trp284, Ser285, Trp287 and Ser288 residues are all conserved in known CBDs. The Leu100, Leu108, Val111, Ala127, Leu129, Val131, Leu159, Tyr169, Val171, Met173, Val175, Phe189, Gly191, Ile194, Leu195, Pro197, Ile203, Val205, Leu215, Val217, Leu232, Phe234, Leu236, Tyr238, Phe246, Trp249, Ile260, Ala263, Val271, Leu273, and Glu286 residues are mainly conserved hydrophobic residues in known CBDs. The Pro98, Pro117, Trp225, Cys258, His269, Ala291 and Gly293 are, in the majority, conserved residues in all known CBDs.

Figure 3(b) depicts the sequence alignment of the CBDs from IL-6R, IL-11R, PRLR and GCSR. Loops L1 to L7 are outlined by boxes.

Figure 4. The CBD of IL-6R with domain D3 (lower part - shade 1) and domain D2 (top part - shade 2), with the loop residues from D3 (shade 3) and from D2 (shade 4). Shades 1 to 4 are of increasing darkness. (a) and (c) have CPK and loop representations of the cytokine binding region loops L1 to L7. (b) and (d) are the same as in (a) and (c) but rotated 90° with the loops facing up.

Figure 5. Comparison of the sequences of CBDs from 77 known genes. Figure 5A compares the sequences in the "first" FnIII domain, containing loops 1 to 4, and Figure 5B the sequences in the "second" FnIII domain, containing the loops 5-7. Conserved residues as described in Example 3 for the IL-6 receptor are aligned according to their sequence homologies. For example the hydrophobic residues, the cysteine residues (C) and in some cases two prolines side by side (PP) are aligned. The location of the 7 binding loops is indicated by the double-headed arrows..

Figure 6. The backbone of the CBD of IL-6R, with the cytokine binding loops L1 to L7 coloured dark. In (a) and (b) a CPK representation the residues that are conserved in all known CBDs. In (c) and (d) including a CPK representation of all residues which are almost always conserved and mainly hydrophobic.

Figure 7. Pictorial representation of the scaffold, firstly demonstrating the structural similarities of the IL-6R, prolactin receptor and the novel scaffold, and secondly the close structural alignment of all three as shown in the central picture.

Detailed description of the invention

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in molecular biology and biochemistry). Standard techniques are used for molecular and biochemical methods (see generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 3rd ed. (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. - and the full version entitled Current Protocols in Molecular Biology, which are incorporated herein by reference) and chemical methods.

Throughout the specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

By "hydrophobic residues" or "nonpolar residues" as used herein is meant valine, leucine, isoleucine, methionine, phenylalanine, tyrosine, and tryptophan.

By "polar residues" herein is meant serine, threonine, histidine, aspartic acid, asparagine, glutamic acid, glutamine, arginine, and lysine.

By "extracellular domain" of as used herein is meant a segment of a protein existing predominantly outside the cell. For transmembrane proteins, this segment can be tethered to the cell through a transmembrane domain or released from the cell through proteolytic digestion. Alternatively, the extracellular domain could comprise the whole protein or amino acid segments thereof when secreted from the cell.

Cytokine binding domains (CBDs)

A cytokine binding domain is defined herein as a polypeptide consisting of a first and a second FnIII-like domain. The FnIII-like domains are each independently domains having immunoglobulin folds in a FnIII-like association of beta sheets. The two domains lie on a similar plane and are typically connected at about 90° to each other. Preferably, at least one domain comprises a tryptophan-arginine ladder region, which preferably comprises a Trp-Ser-X-Trp-Ser ("WSXWS") motif or variant thereof which forms a left-handed 3_{10} helix.

Each FnIII-like domain comprises a number of loops, typically surface and solvent exposed loops. The loops in the two domains making up the CBD are arranged in a substantially linear manner over the two domains to form, and to substantially define, a binding region.

The structural definition of CBDs given above is further illustrated and supported by reference to Figures 1-6. In particular, it is further illustrated and supported by reference to the primary, secondary and tertiary structure, including the three dimensional structure, of IL-6R as presented in Figures 1-6 and detailed in Varghese JN *et al.,* 2002, PNAS 99(25):15959-15964 and PCT/AU02/01255, the entire contents of which are herein incorporated by reference. These references also provide the atomic coordinates of the extracellular domain of IL-6R. Figures 1-6 and the aforementioned references variously provide details of structural features, including the arrangement of beta sheets, the orientation of each of the two domains with respect to one another and the location of the solvent exposed loops that are typically present in CDBs.

The amino acid sequence of IL-6R is given in Figure 3, which also highlights the location of various secondary structures in the primary sequence. The CBD of IL-6R is defined by the D2 and D3 domains (amino acids 92 to 297). The two domains lie on a similar plane to form a long flat structure in which the D2 and D3 domains are connected at about 90° to each other. The D2 domain comprises 4 solvent exposed loops (L1: Lys105 to Asn110; L2: Lys133 to Glu140; L3: Ala160 to Phe168; and L4: Gln190 to Gly193) and the D3 domain comprises 3 solvent exposed loops (L5: Asn226 to Arg233; L6: Met250 to His256; and L7: Gln276 to Gln281), which together form a long and narrow binding area held in place by the rigid D2 and D3 framework of the CBD. The location of these loops in the three-dimensional structure of folded IL-6R is shown in Figure 4.

Arg239, Phe246, Arg237, Trp287, Arg274, Trp284 and Gln276 together form the tryptophan-arginine ladder region, which comprises a WSXWS motif.

The alignment of CBDs present in over seventy gene products is shown in Figure 5. Figure 5A depicts the sequence alignment of the first FnIII-like domain (corresponding to D2 of the IL-6R CBD), defined over location R1 to approximately R180 as numbered in Figure 5. Figure 5B depicts the sequence alignment of the second FnIII-like domain (corresponding to D3 of the IL-6R CBD), defined over location approximately R185 to R299 as numbered in Figure 5. The hinge connecting the first and second FnIII-like domains is defined over the approximate location of R180 to R185, e.g. from R181 to R184, as numbered in Figure 5. The hinge region typically comprises residues flanking the side of loop L4.

The alignments in Figures 5A and 5B clearly demonstrate a high degree of conservation. For example, cysteine residues, hydrophobic amino acid residues, hydroxylated amino acid residues, proline/glycine residues, acidic amino acid residues and basic amino acid residues are all variously conserved. Examples of conserved amino acid residues found in the alignments of Figure 5 are given in Table 1.

Table 1: Examples of conserved amino acid residues found in the alignments of Figure 5.

Conserved residues	**Location in Figure 5**	**FnIII-like domain**
Cys	R25, R46, R91, R115	First
Hydrophobic	R22, R26, R41, R44, R48, R64, R66, R117, R136, R138, R140, R142, R146, R156, R158, R161, R162, R170, R172	First
	R187, R189, R191, R197, R208, R210, R212, R214, R224, R227, R280, R282, R285, R287, R295, R297, R299, R319, R322, R326, R328	Second
Hydroxylated (Tyr, Thr, Ser and including His)	R47, R62, R64, R68, R70, R94, R136	First
	R210, R214, R203, R320, R323, R330	Second
Pro/gly	R14, R15, R18, R50, R51, R164, R166, R167	First
	R185, R193, R195, R198, R199, R216, R218, R177, R289, R290, R317, R324, R325	Second
Acidic	R211, R321	Second
Basic	R298	Second

Table 1 is not intended to be a comprehensive analysis of the degree of conversation across the CBD sequences shown in Figure 5. It merely indicates some of the positions where conservation is occurring and serves to demonstrate the extent of conservation. The skilled person will appreciate that there are other positions and complexities of conservation present in the aligned sequences in Figure 5 and will be able to elucidate these using knowledge and analytical tools that are routinely available to them.

Figure 5 also demonstrates that certain motifs, such as the WSXWS motif, are present in the vast majority of CDBs (see, for example, location R321-R325). Particularly significantly, all the sequences have 7 loops corresponding to loops L1 to L7 identified and discussed above in relation to IL-6R above. Table 2 details the approximate locations of these loops as found in Figure 5. It will be understood that loops may also comprise one or more amino acids flanking the locations in Figure 5 as defined in Table 2.

Suitably, the loops may comprise up to 10, preferably up to 5 and more preferably up to 4 flanking amino acids

Table 2: Location of loops L1 to L7 in Figure 5.

Loop	Location in Figure 5	FnIII-like domain
L1	R28-43	First
L2	R70-87	First
L3	R118-135	First
L4	R157-160	First
L5	R198-209	Second
L6	R228-278	Second
L7	R300-316	Second

It will be understood that FnIII-like domains may be derived from proteins not specifically disclosed herein. Furthermore, the skilled person will have no difficulties identifying such other suitable FnIII-like domains within CBDs from other proteins. A number of methods have been described for identifying protein sequences of suitable structure and function. These methods include, but are not limited to, sequence alignment methods, structure alignment methods, sequence profiling methods and energy calculation methods. It is evident from the alignments presented in Figure 5 and from structural information and published crystallographical data (for example Aritomi M. *et al., Nature*, 1999, 401(6754):713-7; Bravo J. *et al., EMBO J.*, 1998, 17(6):1665-74; Elkins P.A. *et al.,* Cell, 1999, 97(2):271-81; Josephson K. *et al., Immunity*, 2001, 15(1):35-46; Man D. *et al., J. Biol. Chem.*, 2003, 278(26):23285-94; Schreuder H. *et al., Nature*,1997, 386(6621):194-200) that the CBD structure exemplified by IL-6R is conserved in other CBDs. Thus, CBDs can be defined with reference to the three-dimensional structure of domains D2 and D3 of IL-6R, in particular with reference to the structural coordinates of the backbone carbon atoms of IL-6R as provided in Varghese JN *et al.,* 2002 PNAS 99(25):15959-15964 and PCT/AU02/01255. Thus, as new crystal structures are solved, it will become immediately apparent if a protein contains a CBD comprising FnIII-like domains by comparing sequence and structural (secondary and tertiary) data with, for example, that of IL-6R and other proteins listed in Figure 5. However, it will be appreciated that the three-dimensional structure of other CBDs will not correspond precisely to that of the IL-6R. Figure 6 illustrates in the context of the IL-6R, the regions of the CBD structure that are most highly conserved in known naturally occurring CBDs.

Alternatively and/or additionally, suitable CBDs may be identified through sequence alignment analysis with the sequences in Figure 5. It will be readily apparent to the skilled person upon carrying out a suitable alignment analysis whether the protein comprises a CBD having two FnIII-like domains. The amino acid sequence of a potential CBD can be directly compared with the sequences in Figure 5 and in particular those residues known to be highly conserved for known CBDs as described above. After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e. avoiding the elimination of conserved residues through arbitrary deletion and insertion), any residues equivalent to particular conserved amino acids in the sequences of Figure 5 should become defined. Furthermore, any sequence motifs should also be identified as should regions where loop structures are likely to occur (i.e. regions where there is little or no predicted secondary structure and which are relatively polar in nature).

Suitable computational methods for carrying out such analyses to identify protein sequences having the desired structural and functional properties are well known in the art and include, for example, Modeller.

Preferably, the first FnIII-like domain of the CBD comprises four loops located at positions L1 to L4 as indicated in Figure 5A when the amino acid sequence is aligned with the sequences in Figure 5.

Preferably, the second FnIII-like domain of the CBDs of the present invention comprises three loops located at positions L5 to L7 as indicated in Figure 5B when the amino acid sequence is aligned with the sequences in Figure 5.

Preferably, the second FnIII-like domain comprises a tryptophan-arginine ladder region, which preferably comprises a WSXWS motif or variant thereof.

Preferably, the first FnIII-like domain comprises four loops located at positions L1 to L4 as indicated in Figure 5A and the second FnIII-like domain comprises three loops located at positions L5 to L7 as indicated in Figure 5B when the amino acid sequence is aligned with the sequences in Figure 5.

The presence of loops L1 to L4 and L5 to L7, and, if present, a tryptophan-arginine ladder would be evident from a suitably performed sequence alignment and analysis.

As an alternative to Figure 5, it is also possible to identify suitable CBDs through homology of the primary sequence with Figure 3 in the same way as described above in relation to Figure 5.

Where crystal structure data is not available, computer modelling tools are now routinely available that allow potentially useful CBD candidates to be modelled and their predicted structures to be directly compared with, for example, the CBD of IL-6R. Therefore, in addition to being able to identify whether a protein contains two FnIII-like domains presenting the loops identified in Figure 5 at analogous positions along the

primary sequence and preferably possessing other motifs such as a tryptophan-arginine ladder region, which preferably comprises a WSXWS motif or variant thereof, it is also possible for the tertiary structure of the protein, or at least the relevant domain of the protein, to be computer modelled and that 3-D model compared with known crystal structures of CBDs, such as the IL-6R CBD. In this way, the spatial correlation of the loops in the protein of interest can be compared with that in known CBDs.

Although Figures 1, 2, 3, 5 and 6 mention seven loops, it will be understood that the loop given as L4 (corresponding to A190 to G193 of IL-6R and located at R154-R160 in Figure 5) is small and in some literature may not always be referred to as a loop *per se*. It has been included in the present description for the sake of completeness. However, this does not mean that the present invention excludes CBDs described in the literature as comprising six loops. On the contrary, such CBDs may evidently be within the scope of the present invention.

The FnIII-like domains of the CBDs of the binding moieties may be derived from any suitable naturally occurring CBDs. Examples of suitable naturally occurring CBDs are listed in Figure 5. Preferably, the CBDs are derived from the extracellular domains of growth factor and cytokine receptor family members, and in particular cytokine receptor family members and associated proteins such as, for example, gp130. Preferred cytokine receptor family members are those in class I (hematopoietin receptors) or class II, preferably class I. Examples of suitable proteins from which CBDs may be derived include the IL-Rs (interleukin receptors), G-CSFR (granulocyte colony stimulating factor receptor), GM-CSFR (granulocyte macrophage colony stimulating factor receptor), PRLR (prolactin receptor), LIFR (leukemia inhibitory factor receptor), OSMR (oncostatin M receptor), cardiotrophin CT-1 receptor, CNTFR (ciliary neutrotrophic factor receptor), leptin receptor, EPOR (erythropoietin receptor), gp130, GHR (growth hormone receptor) and stromal lymphopoietin protein receptor. The numbering of the amino acid residues that constitute the CBD for many of these proteins is provided in Figure 5.

Examples of suitable IL (interleukin) receptors include the IL-2R, IL-3R, IL-4R, IL-5R, IL-6R, IL-7R, IL-9R, IL-11R, IL-12R, IL-13R, IL-15R and IL-21R.

For the avoidance of doubt, with regards to cytokine receptors having alpha and beta subunits, any extracellular domains referred to herein from which suitable CBDs may be derived are alpha subunit extracellular domains, not beta subunit domains.

The FnIII-like domains of a CBD of the invention can be derived from the same or different sources. For example, the first FnIII-like domain may be derived from one protein and the second FnIII-like domain derived from a different protein. For example, the first domain of IL-11R could be combined with the second domain of IL-12R. Similar pairing could also be performed with IL-5R and IL-4R and with prolactin and GMCSFR. Where the two FnIII-like domains are derived from different proteins, it will be

appreciated by the skilled person that they must be suitably orientated with respect to each. The first FnIII-like domain should be suitably hinged to the second FnIII-like domain so that the domains lie in a similar plane, the domains being orientated with respect to each other as they would be to their respective other FnIII-like domain in the native protein from which they derive.

Linkers used to link protein domains are well-know and well understood in the art, in particular in relation to proteins in the immunoglobulin superfamiles. Therefore, the skilled person will appreciate that any suitable hinge may be used to connect the two FnIII-like domains. The two FnIII-like domains can be linked by genetic or chemical means. Examples of suitable chemical linkage include linking the two domains using a suitable cross-linker such as dimaleimide. Alternatively, the two domains may be linked by providing cysteine residues at the respective C- and N-terminals and forming a disulphide bond. In addition, they could be linked using single chain GlySer linkers such as GlyGlyGlyGlySer.

The domains may also be linked genetically. For example, where a restriction enzyme (RE) site naturally occurs between loops 4 and 5 in a wild type CBD, this site can be used to link the two domains. Alternatively, a suitable RE site may be introduced between loops 4 and 5. Preferably, any RE site will lie between that part of the sequence encoding the region of the FnIII-like domains between the end of the beta sheet immediately following loop 4 and the beginning of any beta sheet immediately preceding loop 5.

Figure 5 presents numerous examples of naturally occurring hinges in CBDs. Preferably, the hinge is a stretch of from about 3 to 15 amino acids, preferably from about 4 to 10 amino acids, situated between the two FnIII-like domains. The hinge connects loop 4 to loop 5 via the respective N- and C-terminals of the two domains. Preferably, the hinge is derived from one of the sources from which one of the FnIII-like domains is derived.

It will be apparent that the binding moieties of the invention can be generated *de novo* based on the structural constraints for a CBD described here and above.

In a preferred embodiment, the two FnIII-like domains of a CBD are derived from the same source protein.

Binding Moieties

The binding moieties of the present invention comprise an extracellular CBD consisting of a first FnIII-like domain and a second FnIII-like domain in which the CBD comprises a modification which alters at least one property of the CBD. It will be understood that the binding moieties of the present invention do not encompass and do not relate to the full-

length, wild-type proteins from which suitable FnIII-like domains may be derived. Rather, they encompass and relate to portions of CBD-containing receptors, preferably the extracellular portions, which have been removed or isolated from their natural environments. Where the binding moieties are derived from the extracellular portion of a CBD-containing receptor, the binding moieties are preferably no larger in terms of the number of amino acid residues and/or molecular weight than the native extracellular domain from which the FnIII-like domain(s) is/are derived.

In a preferred embodiment, the CBD of the binding moiety accounts for at least 50%, preferably at least 60%, more preferably at least 70%, yet more preferably at least 80%, even more preferably at least 90% and most preferably at least 95% of the total molecular weight of and/or number of amino acid residues in the binding moiety. In a particularly preferred embodiment, the binding moiety consists essentially of the CBD.

Preferably, the only binding domains present in the binding moieties of the present invention are the two FnIII-like domains. The two FnIII-like binding domains form a single binding region. The binding moieties of the present invention are therefore monomeric polypeptide or protein bodies.

Altered Properties

The CBD is modified such that a property of the CBD is altered.

A property of a cytokine binding domain is altered if any characteristic or attribute of the cytokine binding domain differs from the corresponding property of the unmodified cytokine binding domain. These properties include, but are not limited to, substrate specificity, substrate affinity, binding affinity, binding selectivity, catalytic activity, thermal stability, alkaline stability, pH activity profile, resistance to proteolytic degradation, kinetic association, kinetic dissociation, immunogenicity, ability to be secreted, ability to activate receptors, ability to treat disease, solubility, cytotoxic activity and oxidative stability.

Unless otherwise specified, a property of a cytokine binding domain is considered to be altered when the property exhibits at least a 5%, preferably at least 10%, more preferably at least a 20%, yet more preferably at least a 50%, and most preferably at least a 2-fold increase or decrease relative to the corresponding property in the unmodified cytokine binding domain.

In a preferred embodiment, the solubility of the modified CBD, and concomitantly the binding moiety, is altered, preferably improved, relative to the corresponding unmodified CBD (i.e. the unmodified binding moiety).

In another preferred embodiment, the stability of the CBD is altered, preferably improved, relative to the corresponding unmodified CBD. Examples of altering the

stability include changing one of the following properties:- thermal stability, alkaline stability, pH activity profile and resistance to proteolytic degradation.

In a particularly preferred embodiment, the binding characteristics of the CDB are altered. Examples of altering the binding characteristics include changing one of the following properties: substrate specificity, substrate affinity, catalytic activity, kinetic association, kinetic dissociation, binding affinity and binding selectivity.

Modifications

By modifying the cytokine binding domain we mean introducing at least one modification into a wild type FnIII domain from a wild type cytokine binding domain sequence.

By "wild-type cytokine binding domain" we mean a cytokine binding domain that is found in nature and includes allelic variations; that is, an amino acid sequence that has not been intentionally modified. The wild type cytokine binding domain sequence may be derived from any species, preferably a mammalian species. In a preferred embodiment, the wild-type cytokine binding domain has a sequence as shown in Figure 5.

Suitable modifications include substitutions, insertions and deletions within at least one specified region.

Preferably, the size and/or area of the CBD is altered as compared with the unmodified CBD. Preferably, at least 1, preferably at least 2, more preferably at least 3, 4 or 5, and yet more preferably at least 10 amino acids of a CBD are modified. Modifications can be made to a number of regions.

In a preferred embodiment, a solvent exposed region is modified and, preferably, a number of such regions are modified. Preferred solvent exposed regions are the loops of the CDB. Suitably, modifications are made to alter the size and/or area of a loop, preferably to increase the size and/or area of the loop. The size may suitably be increased by at least 1, 2, 3, 4 or 5 amino acids and preferably by at least 10 or 20 amino acids. A loop size may be increased by up to as many as 40, or even maybe as many as 50 amino acid residues. Modifications can be made to any of the L1, L2, L3, L4, L5, L6 and L7 loops as defined by IL-6R and/or Figure 5. Suitably, modifications are made to at least two or three different solvent exposed regions, e.g. to at least two or three of any the L1, L2, L3, L4, L5, L6 and L7 loops. The solvent exposed regions can be modified by insertion, substitution or by other suitable modifications described herein.

For example, loop L1 in IL-6R is positioned in the centre of the CBD (Figures 1, 2, 4 and 6). Since loop L1 of the CBD of IL-6R contains a natural disulphide bond, this might constrain the flexibility and so form an ideal semi-rigid scaffold for the display of larger, protruding 'finger-like' loops by insertion of additional amino acids within the L1 loop. These protruding 'finger-like' loops are then likely to provide a complementary

binding surface to cavities within the target antigen (protein) to which the CBD is capable of binding, analogous to the protruding loops observed in natural camelid VhH and shark NAR domains (Muyldermans S *et al.,* 2001 Trends Biochem Sci. 26(4):230-5) and (Nuttall SD *et al.*, 2000 Curr Pharm Biotechnol. 1(3):253-63).

Also encompassed are modifications which are essentially tantamount to conservative substitutions throughout the sequence but which alter a property of the CBD. Such conservative substitutions are shown in Table 3.

Table 3: Exemplary conservative substitutions.

Original Residue	**Exemplary Substitutions**
Ala (A)	val; leu; ile; gly
Arg (R)	lys
Asn (N)	gln; his;
Asp (D)	glu
Cys (C)	ser
Gln (Q)	asn; his
Glu (E)	asp
Gly (G)	pro, ala
His (H)	asn; gln
Ile (I)	leu; val; ala
Leu (L)	ile; val; met; ala; phe
Lys (K)	arg
Met (M)	leu; phe;
Phe (F)	leu; val; ala
Pro (P)	gly
Ser (S)	thr
Thr (T)	ser
Trp (W)	tyr
Tyr (Y)	trp; phe
Val (V)	ile; leu; met; phe, ala

Furthermore, if desired, non-naturally occurring amino acids or chemical amino acid analogues can be introduced as a substitution or addition into the polypeptide of the present invention. Such amino acids include, but are not limited to, the D-isomers of the common amino acids, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, 2-aminobutyric acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino

propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogues in general.

Also provided by the invention are chemically modified derivatives of CBDs which may provide advantages such as increasing stability and circulating time of the polypeptide, or decreasing immunogenicity (see U.S. Pat. No. 4,179,337). The chemical moieties for derivitization may be selected from water-soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like.

Also included are binding moieties which are differentially modified during or after synthesis, e.g., by biotinylation, benzylation, glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, etc. The CBDs may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties. These modifications may, for example, serve to increase the stability and/or bioactivity of the binding moieties of the invention.

The CBDs may also be modified by having carboxy-terminal truncations. However, the scope for such modifications is limited and it is preferred that no more than 8 residues, more preferably not more than 6 residues, of the last beta strand in the FnIII-like domains is removed. Preferably, there is no truncation in the first FnIII-like domain.

Altering binding characteristics

In a preferred embodiment, the modification alters the binding characteristics of the CBD. The cytokine binding region which normally contacts the natural ligand for the CBD is typically the solvent exposed region of the CBD and is generally made up of the surface exposed loops. For example, domains D2 and D3 of IL-6R together comprise 7 cytokine binding loops (L1 to L7), as described above. The location of these loops in other CBDs is shown in Figure 5. Thus it is preferred that modifications are made to one or more of these loop regions, or the equivalent regions in other CBDs, in order to alter the binding characteristics.

For example, the binding affinity of the CBD for at least one of its natural ligands can be reduced or abolished. Preferably at least a two-fold, more preferably at least a five- or ten-fold reduction in binding affinity for at least one natural ligand is achieved.

In one embodiment, the binding specificity of the modified CBD is different to that of the unmodified CBD. Preferably, the unmodified CBD is derived from the extracellular

domain of a first receptor having specificity for a first ligand and one or more of the loops of the unmodified CBD having been replaced with the corresponding one or more loops from a second receptor having specificity for a second ligand with the result that the modified CBD has a specificity for the second ligand. For example, the binding specificity of the CBD could be altered to a different cytokine. In particular, this can be achieved by replacing the loops in the cytokine binding region of a CBD which has specificity for a first cytokine, with the loops from a cytokine binding region of a second CBD which has specificity for a second cytokine. For example, the loops L1 to L7 of the CBD of IL-6R could be replaced by loops L1 to L7 of the CBD of IL-11R to provide the modified binding moiety with specificity for IL-11 instead of IL-6. Similarly, the loops L1 to L7 of the CBD of IL-6R could be replaced by loops L1 to L7 of the CBD of prolactin receptor, LIF receptor or oncostatin M receptor to provide the modified binding moiety with specificity for prolactin and/or growth hormone, LIF or oncostatin M respectively instead of IL-6.

In a preferred embodiment, the first receptor is the IL-6R and the second receptor is either prolactin receptor, LIF receptor or oncostatin M receptor, thus altering the ligand specificity of the CBD from IL-6 to either prolactin and/or growth hormone or LIF or oncostatin M, respectively.

In an alternative preferred embodiment, the first CBD is prolactin receptor, or IL-11R, or CNTF receptor which has been modified such that the loops of the cytokine binding region have been replaced with the loops of a second cytokine receptor region alters the specificity of the first CBD.

Modifications can also be made to regions of the CBD that are not solvent exposed and/or which do not form part of a cytokine binding loop (i.e. L1 to L7). For example, the binding moiety may comprise one or more modifications to the hinge region of the CBD and/or to the binding interface of the FnIII-like domains of the CBD. Modifications to the binding interface between the two FnIII-like domains may result in an altered geometry of the spatial relationship between the two domains . This in turn can be used to alter the orientation and/or association of the solvent exposed binding regions, e.g. the loops, which will modify the characteristics/topology of the overall binding surface.

Modifications to the binding interface between the two FnIII-like domains may, for example, involve modifying, either directly or indirectly (e.g. sterically), generally highly conserved hydrophobic residues which are buried and which act to stabilise the association between the two domains. For example, it may involve modifying one or more of residues Pro107, Leu195 and Pro197 of D2 of IL-6R and Trp225, Leu232, Ala275, Pro200 and Pro222 of D3 of IL-6R, or corresponding residues in other CBDs.

Altering physicochemical properties

In a preferred embodiment, a modification alters, and preferably improves, the biophysical and/or physicochemical properties of the binding moiety. Preferably, the modification alters, preferably improves, the stability and/or solubility properties of the binding moiety.

For example, modifications at the domain interface, including interface mutations, can be made to improve surface complementarity. For example, cysteine residue insertions may be made to provide for disulphide stabilisation.

Modifications may also be made to alter, preferably improve, the stability of the scaffold structure. For example, amino acids may be substituted with other amino acids having larger side chains in order to fill out internal holes in the globular structure. Such substitutions could include, for example, glycine to alanine, asparagine to glutamine, aspartate to glutamate, phenylalanine to tyrosine or tryptophan, tyrosine to tryptophan, asparagine or aspartate to histidine, histidine to tyrosine and lysine to arginine. Glycine residues may also be substituted to decrease the flexibility of the protein backbone. In contrast, Proline residues may be inserted or substituted to improve the flexibility of the scaffold, e.g. where there are limitations in the dihedral angles of the protein backbone and in the secondary structure. Other suitable modifications for altering, and in particular improving stability, will be apparent to the skilled person.

In a preferred embodiment, the binding moiety is modified so as to alter, and preferably improve, its solubility as compared with the unmodified binding moiety. A variety of strategies may be employed to improve solubility and in particular design binding moieties that are solubly expressible in cellular hosts (i.e. non-aggregating). For example, modifications can be made that (i) reduce hydrophobicity by replacing solvent exposed hydrophobic residues with suitable polar residues; (ii) increase polar character by replacing neutral polar residues with charged polar residues; (iii) replace non-disulphide bonded cysteine residues (unpaired cysteines) with suitable non-cysteine residues, and (4) replace residues whose identity is different in the corresponding CBD derived from another species (e.g. substitute murine IL-6R residues into human IL-6R). Other alternative strategies will also be apparent to the skilled person. For example, modifications that increase the stability of a protein can sometimes improve solubility by decreasing the population of partially folded or misfolded states. As another example, protein solubility is typically at a minimum when the isoelectric point of the protein is equal to the pH of the surrounding solution. Modifications, which perturb the isoelectric point of the protein away from the pH of a relevant environment, such as serum, can therefore serve to improve solubility.

In a preferred embodiment, one or more, preferably hydrophobic, residues in solvent exposed regions, preferably in a loop, are replaced with structurally and

functionally compatible polar residues. Alanine and glycine may also serve as suitable replacements, constituting a reduction in hydrophobicity.

In an alternate embodiment, preferred polar residues include those that are observed at homologous positions in other CBDs.

In another preferred embodiment, free cysteine residues (that is, cysteine residues that are not participating in disulphide bonds) are mutated to a structurally and functionally compatible non-cysteine residue. Unpaired cysteines can be identified by visual analysis of the structure or by analysis of the disulphide bond patterns of related proteins.

In a preferred embodiment, if the non-disulphide forming cysteine position is substantially buried in the CBD framework, the cysteine may be removed or replaced with, for example, a suitable non-cysteine residue such as alanine or serine. If the cysteine position is substantially exposed to solvent, suitable non-cysteine residues include alanine and the polar residues. Furthermore, cysteine residues not involved in disulphide bond formation within the CBD framework could also be removed or replaced, e.g. with alanines or serines, so as to improve solubility. For example, as regards D2 and D3 of the IL-6R CBD, any one or more of Cys174, Cys192 and Cys258 could be removed, and preferably replaced with serines, to improve solubility.

In a preferred embodiment, one or more solvent exposed loops is/are modified to improve solubility. Solubility may be improved by, for example, either removing disulphide bond-forming cysteines and/or replacing disulphide bond-forming cysteines from within the solvent exposed loops with amino acids such as alanine or serine.

Modifications to improve solubility may be desirable where the binding moieties are being designed to function in an intracellular context and/or their method of production favours expression in a soluble form. It will also be evident to the skilled person that it may be necessary to modify the solubility characteristics of the binding moiety at the same time or even prior to making other modifications, such as, changing the binding characteristics.

The physicochemical properties, such as stability and solubility, of the binding moieties may be qualitatively and/or quantitatively determined using a wide range of methods known in the art. Methods which may find use in the present invention for characterizing the biophysical/physicochemical properties of the binding moieties include gel electrophoresis, chromatography such as size exclusion chromatography, reversed-phase high performance liquid chromatography, mass spectrometry, ultraviolet absorbance spectroscopy, fluorescence spectroscopy, circular dichroism spectroscopy, isothermal titration calorimetry, differential scanning calorimetry, analytical ultra-centrifugation, dynamic light scattering, proteolysis, cross-linking, turbidity measurement, filter retardation assays, immunological assays, fluorescent dye binding assays, protein-staining

assays, microscopy, and detection of aggregates via ELISA or other binding assay. Structural analysis employing X-ray crystallographic techniques and NMR spectroscopy may also find use.

For example, protein stability (e.g. structural integrity) may be determined by measuring the thermodynamic equilibrium between folded and unfolded states.

In one embodiment, stability and/or solubility may be measured by determining the amount of soluble protein after some defined period of time. In such an assay, the protein may or may not be exposed to some extreme condition, for example elevated temperature, low pH, or the presence of denaturant. Because unfolded and aggregated protein is not expected to maintain its function, e.g. be capable of binding to a predetermined target molecule, the amount of activity remaining provides a measure of the binding moieties stability and solubility. Thus, one method of assessing solubility and/or stability is to assay a solution comprising a binding moiety for its ability to bind a target molecule, then expose the solution to elevated temperature for one or more defined periods of time, then assay for antigen binding again.

Alternatively, the modified binding moieties could be expressed in prokaryotic expression systems and the protein isolated from the cell lysate by a series of biochemical purification steps including differential centrifugation, affinity isolation chromatography using attached tags such as poly histidine, ion-exchange chromatography and gel filtration chromatography. A measure of the improvement in the solubility of the modified polypeptide can be obtained by making a comparison of the amount of soluble protein obtained at the end of the purification procedure to that obtained using the unmodified polypeptide, when starting with a similar amount of expressed unfractionated product. Levels of expression of product in culture can be normalised by a comparison of product band densities after polyacrylamide gel electrophoresis of equivalent aliquots of SDS detergent-solubilised cell lysate.

Alternatively, binding moieties can be unfolded using chemical denaturant, heat, or pH, and this transition be monitored using methods including, but not limited to, circular dichroism spectroscopy, fluorescence spectroscopy, absorbance spectroscopy, NMR spectroscopy, calorimetry, and proteolysis. As will be appreciated by those skilled in the art, the kinetic parameters of the folding and unfolding transitions may also be monitored using these and other techniques.

The solubility of the binding moieties of the present invention preferably correlates with the production of correctly folded, monomeric polypeptide. The solubility of the modified binding moiety may therefore also be assessed by HPLC or FPLC, using which soluble (non-aggregated) fragments will give rise to a single peak, whereas aggregated fragments will give rise to a plurality of peaks. A preferred measurement of solubility uses conventional FPLC or HPLC techniques which assess the level of aggregation and

presence of high molecular weight species as described in Power BE et al., 2003, Protein Science 12, 734-747.

As an example of an accelerated stability trial, aliquots of the binding moiety can be stored at different temperatures, such as –20°C, 4°C, 20°C and 37°C and the activity of the binding moiety assayed at different time intervals. For example, successful maintenance of activity during storage at 37°C for 12 weeks is roughly equivalent to storage stability for 12 months at 4°C. The trial can also be conducted to compare the effect of different protecting additives in the storage buffer on the stability of the protein. Such additives can include compounds such as glycerol, sorbitol, non-specific protein such as bovine serum albumin, or other protectants that might be used to increase the shelf life of the protein.

In a preferred embodiment, cysteine residues have been removed or replaced within the CBD, preferably from within one or more of the loops. In a further preferred embodiment, cysteine residues have been removed or replaced in one or more loops of one FnIII-like domain whilst remaining unaltered in the other FnIII-like domain.

It will be understood that any one or more of the type of modifications described above in relation to altering a particular property of a binding moiety may be used to alter other properties in addition to or instead of those which are specifically described in relation to that modification above.

Binding moieties of the invention may be in a substantially isolated form. It will be understood that the protein may be mixed with carriers or diluents which will not interfere with the intended purpose of the protein and still be regarded as substantially isolated. Binding moieties of the invention may also be in a substantially purified form, in which case they will generally comprise the protein in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the protein in the preparation is a binding moiety of the invention.

The binding moieties of the invention may be linked to other molecules, for example by covalent or non-covalent means. In preferred embodiments, the binding moieties (CBD) of the invention may be linked (without restriction) to molecules such as enzymes, drugs, lipids, sugars, nucleic acids and viruses.

In one embodiment, the binding moiety may contain solvent exposed cysteine residues for the site-specific attachment of other entities.

Binding moieties of the invention can be linked to other molecules, typically by covalent or non-covalent means. For example, binding moieties may be produced as fusion proteins, linked to other polypeptide sequences. Fusion partners can include enzymes, detectable labels and/or affinity tags for numerous diagnostic applications or to aid in purification. Fusion partners, without restriction, may be GFP (green fluorescent protein), GST (glutathione S-transferase), thioredoxin or hexahistidine. Other fusion

partners include targeting sequences that direct binding moieties to particular sub-cellular locations or direct binding moieties to extracellular locations e.g. secretion signals. In a preferred embodiment binding moieties of the invention do not comprise other regions of the receptor/protein from which they are derived i.e. any fusion partners are heterologous to the CBD. The heterologous sequence may be any sequence which allows the resulting fusion protein to retain the activity of the modified CBD. The heterologous sequences include for example, immunoglobulin fusions, such as Fc fusions, or fusions to other cellular ligands which may increase stability or aid in purification of the protein.

Diagnostic or therapeutic agents that can be linked to the binding moieties of the invention include pharmacologically active substances such as toxins or prodrugs, immunomodulatory agents, nucleic acids, such as inhibitory nucleic acids or nucleic acids encoding polypeptides, molecules that enhance the *in vivo* stability or lipophilic behaviour of the binding moieties such as PEG, and detectable labels such as radioactive compounds, dyes, chromophores, fluorophores or other imaging reagents.

Binding moieties may also be immobilised to a solid phase, such as a substantially planar surface (e.g. a chip or a microtitre plate) or beads. Techniques for immobilising polypeptides to a solid phase are known in the art. In addition, where libraries of binding moieties are used (e.g. in screening methods), arrays of binding moieties immobilised to a solid phase can be produced (Lee YS and Mrksich, M, 2002 Trends Biotechnol. 20(12 Suppl):S14-8. and references contained therein).

In another embodiment of the invention, the binding moieties of the invention function as a protein scaffold with other polypeptide sequences being inserted into solvent-exposed regions of the binding moiety for display on the surface of the scaffold. Such scaffolds may, for example, serve as a convenient means to present peptides in a conformationally constrained manner. The scaffolds may be used to produce CBDs with altered binding specificities and also to produce and/or screen for binding moieties having specificity for any target molecule of interest.

Heterologous polypeptide sequences may be inserted into one or more solvent exposed regions such as, for example, one or more loops of the CBD. The CBD of the binding moiety functions as a protein scaffold for the inserted heterologous sequences, displaying the heterologous sequences on the surface of the binding moiety.

The heterologous sequences may replace all or part of the loop of the CBD into which they are inserted, or may simply form additional sequence. Preferably, a plurality of heterologous sequences are inserted into a plurality of loops.

The heterologous sequences may be derived from solvent exposed regions such as, for example, loops of another CBD. They may also be derived from other non-CBD molecules or be partially of fully randomised.

Other modifications can also be made to the scaffold proteins of the invention as described in the previous sections in relation to CBDs and they may also be linked to other molecules and/or produced as multimers as described below.

Two or more CBDs may be joined together to form multimers through either covalent linkage or non-covalent linkage or a combination of linkages, including the use of chemical or genetically-encoded linkers. CBD multimers are one preferred design for therapeutic reagents since they have the potential to provide increased avidity and slower blood clearance rates which may provide favourable pharmacokinetic and biodistribution properties. The linkages used are well known to persons skilled in the art, for example in relation to antibodies and antibody fragments joined by chemicals (Casey JL *et al.,* 2002 Br J Cancer. 86(9):1401-10), linkages is by way of genetically-encoded linker polypeptides (BITE's scFv-scFv), or adhesive fusion-domains (Plückthun, A., and Pack, P 1997. Immunotechnology 3, 83-105). Indeed, two FnIII-like domains from different CBDs may be cross-paired using linker polypeptides to form tightly-associated CBD multimers in the manner of a diabody (an antibody Fv dimer) or triabody (antibody Fv trimer) or tetrabody (antibody Fv tetramer) (Power BE et al., 2001, Cancer Immunol Immunother. 50(5):241-50). The resulting CBD multimers from any of these linker strategies described above may possess the same, or different target specificities thus providing multivalent or multispecific reagents. In a preferred embodiment, two CBDs may be joined to form a dimer through either covalent linkage or non-covalent linkage or a combination of linkages thereby providing two target binding affinities. If two or more CBDs in the multimer have the same target specificity, the CBD multimer will be multivalent and have increased avidity (functional affinity) for binding to two or more target molecules.

CBD multimers may be designed to have increased stability by modification to the interface contact regions, either through chemical or genetic alterations. For example, detailed examination of the CBD framework regions at the multimer interface may direct introduction of residue mutations or chemical modifications that stabilise the interface and thereby direct the preferential formation of CBD multimers. In one embodiment, the mutations are introduced to interface residues other than F134(D2), F168 (D2) and H261 (D3). In another embodiment, the mutation is introduced at residue C174 (D2), C192 (D2) or C258 (D3).

Production of binding moieties

Binding moieties of the invention may be made by chemical or recombinant means. Techniques for chemically synthesising peptides are reviewed by Borgia and Fields, 2000, TibTech 18: 243-251 and described in detail in the references contained therein. Typically

binding moieties of the invention are made by recombinant means. Accordingly, the present invention provides polynucleotides encoding binding moieties of the present invention.

Modifications to binding moieties of the invention can be made using standard cloning techniques known to persons skilled in the art, such as site-directed mutagenesis. Variation in the amino acid sequence of a natural unmodified loop or loops can be achieved by designing the encoding gene to produce either specific point mutations or by random 'window' mutagenesis to randomise the entire loop sequence(s) during the construction of a library repertoire. Variation in loop length may be achieved by designing the encoding gene to remove some of the amino acids in the CBD loops, thus making shorter loops or conversely by increasing the number of amino acids to extend the loops. These designs can be applied to two or more loops selected from L1, L2, L3, L4, L5, L6 and L7 loops. Alternatively the entire gene repertoire comprising the CBD framework and the randomised loops can be constructed using synthetic oligonucleotide primers.

One approach to obtaining binding moieties having a binding affinity for a target molecule of interest is to produce libraries of polynucleotides which encode different binding moieties of the invention comprising modifications in the CBR, preferably in one or more loops, and screen the libraries for binding to the target molecule using standard techniques such as phage display or ribosomal display. This screening approach will be described in more detail below.

Polynucleotides, vectors and hosts

Polynucleotides of the invention may comprise DNA or RNA. They may be single-stranded or double-stranded. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modifications to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of the invention.

Polynucleotides of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian cell lines and other eukaryotic cell lines, for example insect Sf9 cells.

Preferably, a polynucleotide of the invention in a vector is operably linked to a control sequence that is capable of providing for the expression of the coding sequence by a host cell or using an *in vitro* transcription/translation system, i.e. the vector is an expression vector. The term "operably linked" means that the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

The control sequences may be modified, for example by the addition of further transcriptional regulatory elements to make the level of transcription directed by the control sequences more responsive to transcriptional modulators.

Vectors of the invention may be transformed or transfected into a suitable host cell to provide for expression of a binding moiety of the invention. This process may comprise culturing a host cell transformed with an expression vector under conditions to provide for expression by the vector of a coding sequence encoding the binding moiety, and optionally recovering the expressed binding moiety.

The vectors may be, for example, plasmid, phagemid or virus vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used, for example, to transfect or transform a host cell.

Control sequences operably linked to sequences encoding the protein of the invention include promoters/enhancers and other expression regulation signals. These control sequences may be selected to be compatible with the host cell for which the expression vector is designed to be used in. The term "promoter" is well-known in the art and encompasses nucleic acid regions ranging in size and complexity from minimal promoters to promoters including upstream elements and enhancers.

The promoter is typically selected from promoters which are functional in prokaryotic or eukaryotic cells. With respect to eukaryotic promoters, they may be promoters that function in a ubiquitous manner or, alternatively, a tissue-specific manner. They may also be promoters that respond to specific stimuli. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR) promoter, the rous sarcoma virus (RSV) LTR promoter or the human cytomegalovirus (CMV) IE promoter.

It may also be advantageous for the promoters to be inducible so that the levels of expression of the binding moiety can be regulated during the life-time of the cell. Inducible means that the levels of expression obtained using the promoter can be regulated.

In a number of embodiments of the present invention, heterologous sequences are inserted into the binding moieties of the present invention, for example where the binding moieties are used as scaffold sequences. Such modifications are generally made by manipulating polynucleotides of the invention encoding binding moieties of the invention. This may conveniently be achieved by providing cloning vectors that comprise a sequence encoding a CBD which sequence comprises one or more unique insertion sites in one or more regions encoding a solvent exposed region of said cytokine domain, to allow for easy insertion of nucleotide sequences encoding heterologous sequences into the appropriate regions of the CBD.

Each "unique" insertion site typically contains a nucleotide sequence that is recognised and cleaved by a type II restriction endonuclease, the nucleotide sequence not being present elsewhere in the cloning vector such that the cloning vector is cleaved by the restriction endonuclease only at the "unique" insertion site. This allows for easy insertion of nucleotide sequences having the appropriate ends by ligation with cut vector using standard techniques well know by persons skilled in the art. Preferably the insertion site is engineered - i.e. where the CBD is derived from a naturally occurring sequence, the insertion site does not naturally occur in the natural sequence.

Vectors and polynucleotides of the invention may be introduced into host cells for the purpose of replicating the vectors/polynucleotides and/or expressing the binding moiety proteins of the invention encoded by the polynucleotides of the invention. Host cells include prokaryotic cells such as bacterial cells and eukaryotic cells including yeast, fungi, insect cells and mammalian cells.

Vectors/polynucleotides of the invention may introduced into suitable host cells using a variety of techniques known in the art, such as transfection, transformation and electroporation. Where vectors/polynucleotides of the invention are to be administered to animals, several techniques are known in the art, for example infection with recombinant viral vectors such as retroviruses, herpes simplex viruses and adenoviruses, direct injection of nucleic acids and biolistic transformation.

Host cells comprising polynucleotides of the invention may be used to express proteins of the invention. Host cells are cultured under suitable conditions which allow for expression of the binding moieties of the invention. Expression of the binding moieties may be constitutive such that they are continually produced, or inducible, requiring a stimulus to initiate expression. In the case of inducible expression, protein production can be initiated when required by, for example, addition of an inducer substance to the culture medium, for example dexamethasone or IPTG, or inducible expression may achieved through heat-induction, thereby denaturing the repressor and initiating protein synthesis.

Binding moieties of the invention can be extracted from host cells by a variety of techniques known in the art, including enzymatic, chemical and/or osmotic lysis and physical disruption.

Libraries of binding moieties

Binding moieties of the present invention may be provided as libraries comprising a plurality of binding moieties which have different sequences in the CBR. Preferably, the variations reside in one or more loops. These libraries can typically be used in screening methods to identify a binding reagent with an activity of interest, such as affinity for a specific target molecule of interest.

Libraries of binding moieties are conveniently provided as libraries of polynucleotides encoding the binding moieties. The polynucleotides are generally mutagenised or randomised to produce a large number of different sequences which differ at one or more positions within at least one loop.

Mutations can be introduced using a variety of techniques known in the art, such as site-directed mutagenesis. A number of methods for site-directed mutagenesis are known in the art, from methods employing single-stranded phage such as M13 to PCR-based techniques (see "PCR Protocols: A guide to methods and applications", M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White (eds.). Academic Press, New York, 1990). Another technique is to use the commercially available "Altered Sites II *in vitro* Mutagenesis System" (Promega - U.S. Patent N° 5,955,363). Techniques for site-directed mutagenesis are described above. Pluralities of randomly mutated sequences can be made by introducing mutations into a nucleotide sequence or pool of nucleotide sequences 'randomly' by a variety of techniques *in vivo,* including; using 'mutator strains', of bacteria such as *E. coli mutD5* (Low *et al.*, 1996, J Mol Biol 60: 9-68); and using the antibody hypermutation system of B-lymphocytes (Yelamos *et al.*, 1995, Nature 376: 225-9). Random mutations can also be introduced both *in vivo* and *in vitro* by chemical mutagens, and ionising or UV irradiation (Friedberg *et al.*, 1995, DNA repair and mutagenesis. SM Press, Washington D.C.), or incorporation of mutagenic base analogues (Zaccolo *et al.*, 1996 J Mol Biol 255: 589-603). 'Random' mutations can also be introduced into genes *in vitro* during polymerisation for example by using error-prone polymerases (Leung *et al.*, 1989, Technique 1: 11-15).

It is generally preferred to use mutagenesis techniques that vary the sequences present in the cytokine binding region (e.g. the loop sequences) of the CBD, although framework changes may also occur which may or may not be desirable. One method for targeting the cytokine binding region is to provide a plurality of relatively short nucleotide sequences that are partially or fully mutagenised/randomised and clone these sequences into

specific insertion sites in the binding moiety, as described above in relation to scaffold sequences.

Another approach is to synthesise a plurality of random synthetic oligonucleotides and then insert the oligonucleotides into a sequence encoding the binding moiety and/or replace a sequence encoding the binding moiety with the random synthetic oligonucleotides. A suitable method is described in WO97/27213 where degenerate oligonucleotides are produced by adding more than one nucleotide precursor to the reaction at each step. The advantage of this method is that there is complete control over the extent to which each nucleotide position is held constant or randomised. Furthermore, if only C, G or T are allowed at the third base of each codon, the likelihood of producing premature stop codons is significantly reduced since two of the three stop codons have an A at this position (TAA and TGA).

Another approach is to generate the gene repertoire using SOE-PCR (splicing overlap extension polymerase chain reaction) a method known to those in the art. This method is used when no full length gene template is available and the gene repertoire is synthetically assembled.

Oligonucleotide synthesis is performed using techniques that are well known in the art (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, IRL Press at Oxford University Press 1991). Libraries can also be specified and purchased commercially. The synthetic process can be performed to allow the generation of all or most possible combinations over the length of the nucleic acid, thus generating a library of randomised nucleic acids. These randomised sequences are synthesised such that they allow in frame expression of the randomised peptide with any fusion partner.

In one embodiment, the library is fully randomised, with no sequence preferences or constants at any position. In another embodiment, the library is biased, i.e. partially randomised in which some positions within the sequence are either held constant, or are selected from a limited number of possible variations. Thus some nucleic acid or amino acid positions are kept constant with a view to maintaining certain structural or chemical characteristics.

The randomised oligonucleotides can then be inserted into a suitable site and/or replace a suitable sequence encoding a binding moiety.

Generally the library of sequences will be large enough such that a structurally diverse population of random sequences is presented. This ensures that a large subset of 3-D shapes and structures is represented and maximises the probability of a functional interaction.

It is preferred that the library comprises at least 1000 different nucleotide sequences, more preferably at least 10^4, 10^5 or 10^6 different sequences. Preferably, the

library comprises from 10^4 to 10^{10} different sequences. Preferably at least 5, 10, 15 or 20 amino acid residues of the peptides encoded by the nucleotide sequences are randomised.

Typically, the inserted peptides encoded by the randomised nucleotide sequences comprise at least 5, 8, 10 or 20 amino acids. Preferably, they also comprise fewer than 50, 30 or 25 amino acids.

The libraries of polynucleotides encoding binding moieties can be screening using any suitable technique to identify a binding moiety having an activity of interest. For example, to identify a binding moiety that binds to a target molecule of interest, the library of polynucleotides is incubated under conditions that allow for expression of the binding moiety polypeptides encoded by the polynucleotides and binding of the polypeptides to the target molecule assessed. Binding is typically assessed *in vitro* or using whole cell assays.

Suitable techniques for screening the library for binding moieties having an activity of interest include phage display and ribosome display as well as the use of viral vectors, such as retroviral vectors.

The sequence of binding moieties identified in the screen can conveniently be determined using standard DNA sequencing techniques.

Diagnostic/Therapeutic Uses of Binding Moieties

Binding moieties of the invention, including those identified in the screening methods of the invention, may be used in methods of diagnosis/therapy by virtue of their specific binding to a target molecule of interest. Such uses will be analogous to the plethora of diagnostic/therapeutic applications already known in relation to antibodies and fragments thereof. For example, binding moieties of the invention may be used to detect the presence or absence of molecules of interest in a biological sample.

For diagnostic purposes, it may be convenient to immobilise the binding reagent to a solid phase, such as a dipstick, microtitre plate or chip.

As discussed above, binding moieties of the invention when used diagnostically will typically be linked to a diagnostic reagent such as a detectable label to allow easy detection of binding events *in vitro* or *in vivo*. Suitable labels include radioisotopes, dye markers or other imaging reagents for *in vivo* detection and/or localisation of target molecules.

Binding moieties may also be used therapeutically. For example, binding moieties may be used to target ligands that bind to extracellular receptors, such as cytokine receptors, and consequently antagonise the effect of such ligands. Cytokines and their receptors are involved in a wide range of disease processes and consequently modulation

of their activity with specifically designed binding moieties based on CBDs has clear clinical implications.

In addition, binding moieties of the invention may be used, in a similar manner to antibodies, to target pharmacologically active substances to a cell of interest, such as a tumour cell, by virtue of binding to a cell surface molecule present specifically on the tumour cell to which the binding moiety binds specifically.

Administration

Binding moieties of the invention including binding moieties identified by the screening methods of the invention may preferably be combined with various components to produce compositions of the invention. Preferably the compositions are combined with a pharmaceutically acceptable carrier, adjuvant or diluent to produce a pharmaceutical composition (which may be for human or animal use). Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition of the invention may be administered by direct injection. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular, oral or transdermal administration. Typically, each protein may be administered at a dose of from 0.01 to 30 mg/kg body weight, preferably from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

Polynucleotides/vectors encoding binding moieties may be administered directly as a naked nucleic acid construct. When the polynucleotides/vectors are administered as a naked nucleic acid, the amount of nucleic acid administered may typically be in the range of from 1 μg to 10 mg, preferably from 100 μg to 1 mg.

Uptake of naked nucleic acid constructs by mammalian cells is enhanced by several known transfection techniques for example those including the use of transfection agents. Example of these agents include cationic agents (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectam™ and transfectam™). Typically, nucleic acid constructs are mixed with the transfection agent to produce a composition.

Preferably the polynucleotide or vector of the invention is combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, oral, intraocular or transdermal administration.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

The various features and embodiments of the present invention, referred to in individual sections above apply, as appropriate, to other sections, *mutatis mutandis*. Consequently features specified in one section may be combined with features specified in other sections, as appropriate.

EXAMPLE 1: Design of modified IL-6R CBD with altered binding specificity

A PSI_BLAST search of the Brookhaven protein data bank revealed several structures that are closely related to the cytokine binding modules of the human IL-6 receptor. Of these the human prolactin receptor (PRLR) bound to human growth hormone was the most closely related structure that did not have overlapping specificity for interleukin-6. The binding of human growth hormone by the prolactin receptor is mediated by the same loop framework as the cytokine binding modules of IL-6R use to bind IL-6.

Sequence alignment

The sequences of IL-6R and PRLR have been aligned according to their three dimensional structure using the MALIGN3D function of MODELLER6v2.

Loop Definition

Residues from the prolactin receptor in contact with human growth hormone were selected using VMD. VMD is a visualisation package developed at the University of Illinois which allows the viewing and manipulation of large molecules (Schwieters (2001) Journal of Magnetic Resonance 149:239-244). Loop regions were selected to contain these residues and residues which support the correct side-chain orientation of the contact residues.

Homology modelling

The sequence of a CBD binding moiety protein incorporating the framework residues of IL-6R and loop residues from the prolactin receptor was created. An initial series of homology models of the CBD binding moiety was generated using MODELLER6v2 with IL-6R framework residues and prolactin receptor loop residues as templates (see Figure 7). Model quality was assessed using PROCHECK. The loop regions were then refined *ab initio* using MODELLER6v2. Final model was then energy minimised and assessed for stability using CNS (Brünger AT *et al.*, 1998 Acta Crystallog D54:905-921).

EXAMPLE 2: Production of an IL-6R CBD (binding moiety)

Oligonucleotide primers were designed to amplify the CBD domains (the D2 and D3 domains) of human IL-6R by PCR, using IL-6R DNA as a template for this reaction. These PCR fragments of correct size and DNA sequence were cloned into pPOW5 bacterial expression vector. Protein expression was performed using eight different bacterial cell strains. One particular strain was selected for further stability and characterisation studies.

EXAMPLE 3: Modification of an IL-6R CBD to introduce prolactin binding specificity

In another gene construct, the surface loops of prolactin receptor were grafted onto the IL-6R framework to produce a reagent with prolactin binding specificity. The grafting process involved replacement of seven solvent-exposed surface loops L1 to L7 of IL-6R by the equivalent loop residues from prolactin receptor, thereby effectively changing the binding specificity of the modified CBD from IL-6 to prolactin. There are several methods that can result in loop grafting and, in this example, the grafting process involved redesigning the gene encoding the modified IL-6R CBD such that the encoded surface loops L1 to L7 were that of prolactin receptor. The modified CBD gene was then constructed using a gene assembly process using synthetic oligonucleotides, typically 80 bases in length, which were assembled by hybridisation and ligation, into a section of double-stranded DNA encoding the entire modified CBD gene, in an overlapping "brick-laying" fashion. PCR and oligonucleotide primers were used as the final step to amplify the fully assembled gene. The DNA sequence of the PCR product was confirmed, and the modified CBD gene then sub-cloned and expressed in bacteria.

EXAMPLE 4: Producing a novel binding moiety with modified intra-domain disulphide bonds

We produced a binding moiety with a modified intra-domain disulphide bond. We used PCR to introduce a mutation at Cys174 to Ser on the CBD framework. This Cys174 in D2, usually forms a disulphide bond with another cysteine in the first domain of IL-6R (a non-FnIII domain commonly referred to as the D1 domain of IL-6R), and is not involved with the D2 and D3 CBD associations. The Cys174→Ser mutant was subsequently expressed in bacteria.

EXAMPLE 5: Producing a novel binding moiety with no cysteine residues in the D3 domain.

We introduced another CBD framework mutation Cys258 to Serine in domain D3. This is a buried cysteine residue, mutated in an attempt to increase expression and stability of the CBD framework, and to ascertain whether the D3 domain could fold without the need for this Cysteine residue. We have expressed the CBD containing this D3 mutation in bacteria.

Clones isolated from the D3 library also contained this Cys258→to Ser framework mutation (see Examples 7 and 8).

EXAMPLE 6: Producing a novel binding moiety with a removed (replaced) cysteine residues in the solvent exposed region.

We noticed that when the PRLR loop graft onto the IL-6R framework was expressed in bacteria, there were less protein aggregates. There is a solvent exposed Cys192 in the IL-6R framework/loop junction, that is not involved in disulphide bond formation, which is not a cysteine residue in the equivalent position of the PRLR loop. Another mutation Cys192→Ser, which lies at this framework/loop junction was designed within the D2 domain of IL-6R. This is a solvent exposed cysteine in the IL-6R framework and this mutation improved solubility of the IL-6R framework CBD.

EXAMPLE 7: Producing a library repertoire of novel binding moieties based on the CBD scaffold

A gene library comprising the IL-6R CBD was constructed with mutations in the solvent-exposed surface loops. Loops L5, L6 and L7 were mutated in the D3 domain of the CBD by constructing a gene repertoire using overlapping synthetic oligonucleotides and the gene assembly techniques described in Example 3. The overlapping oligonucleotides contained flanking framework residues of IL-6R, then genetic diversity in the loops residues, followed by more framework residues. The genetic diversity encoding the amino acid residues in the loops was biased in such a way as to reduce the chance of stop codons and also to encode for all 20 amino acids at each position of each loop. This diversity was achieved during the synthesis of the degenerate oligonucleotides, wherein instead of adding one nucleotide per position at a time, all four nucleotides (G, A, T and C) were added per position. Stop codons triplets usually end with an A e.g. TAA. The chance of this occurring in the degenerate oligonucleotide was reduced by only allowing G, T and C at the third position of the triplet.

In order to make the genetically diverse library, two different lengths of oligonucleotides were used. The oligonucleotides covering the loop regions were about 80 bases in length (top strand). The reverse oligonucleotide "cementing the bricks" were short, covering only the framework residues, and were about 55 bases in length. PCR was used to fill-in the gaps on the bottom strand. The cloned gene repertoire in the phagemid vector was transformed into bacterial competent cells. Several well-spaced isolated colonies were picked and grown in liquid culture, from which the DNA was extracted and sequenced. The DNA sequence from one of these isolated clones showed mutations within both loop regions as well as the CDB framework.

The IL-6R CBD library framework contained three mutations in which cysteine residues (Cys174, Cysr192 and Cys258) had been replaced by serine residues. In addition to the desired framework changes, the DNA sequence showed changes in loop 6, with residues in that loop being replaced with other residues. This clone was subsequently expressed in bacteria.

The partial DNA sequence of IL-6R D3 (loops 6 and 7 in bold and boxed, and Cys258 in bold) is shown below as sequence (a). The corresponding partial DNA sequence of the D3 library clone, showing changes in loop 6 and at Cys258 (mutated to Ser) shown as sequence (b).

(a) R S K T F T T W **M V K D L** Q H H **C** V I H D A W S G L R H
(b) R S K T F T T W **A Q S R W** Q H H **S** V I H D A W S G L R H

(a) V V Q L R A **Q E E F G Q G** E W S E W
(b) V V Q L R A **Q E E F G Q G** E W S E W

EXAMPLE 8: Producing a novel binding moiety with multi-loop mutations

Another clone isolated from the D3 library described in Example 7 showed changes in both loop 6 and loop 7 residues of the D3 domain. This clone, also containing a CBD framework mutation at Cys258 to Ser, was also expressed in bacteria.

The partial DNA sequence of IL-6R D3 (loops 6 and 7 in bold and boxed, and Cys258 in bold) is shown below as sequence (c). The corresponding partial DNA sequence of the D3 library clone, showing changes in loops 6 and 7 and at Cys258 (mutated to Ser) shown as sequence (d).

(c) R S K T F T T W **M V K D L** Q H H **C** V I H D A W S G L R H
(d) R S K T F T T W **S R Q N D** Q H H **S** V I H D A W S G L R H

(c) V V Q L R A **Q E E F G Q G** E W S E W
(d) V V Q L R A **R N E V R V G** E W S E W

Examples 1 to 8 demonstrate that a functional CBD scaffold can be made from an IL-6R by specific point modifications to improve expression and folding. This was achieved by mutations of Cys174→Ser and Cys192→Ser , in the first domain, with or without mutations of Cys258 in the second domain.

In the first scaffold produced, containing IL-6R loops, the expressed scaffold was isolated by low pH extraction with a citrate buffer. The supernatant was purified by HPLC, collecting the monomer and dimer peaks, separately. The retention times of the monomer and dimer were consistent with expected retention times for these size of molecules. Each peak, when purified, was found to have functional activity as measured using ELISA assays and BIAcore microarrays with the ligand. IL-6 bound to the microtitre plates of the biochip respectively. The results for the association and dissociation constants were indicative of published rates for receptors and their ligands. Furthermore the protein peaks did not bind prolactin ligand, demonstrating that the receptor scaffold maintained its specificity to its ligand.

Examples 1 to 7 also demonstrate the methodology to produce a scaffold library based on IL-6R. This was achieved by introduction of random amino acids in the loop regions through PCR and degenerate codon usage. The repertoire was displayed by construction of a phage display library using a pHFAsacII vector. Individual random clones were isolated. Human target antigens were immobilised onto the surface of magnetic beads using standard amine coupling chemistry. After three rounds of phage panning, isolating binders from each round, the phage pools were then assayed for functional activity using ELISA and BIAcore techniques. Each isolate was also sequenced to determine the DNA sequence.

Having produced a simple scaffold, loop grafting was performed, replacing the IL-6R loops with loops from the prolactin receptor. Successful loop grafting was verified by HPLC, which also showed monomer and dimer protein peaks, which, when purified, were found to contain functional activity. Activity was measured using ELISA assays and BIAcore microarrays, with the IL-6 ligand being bound to the microtitre plates of the biochip. The protein peaks were found to bind prolactin and lactogen as expected. In addition, they also bound IL-6. The modified proteins did however, not bind human growth hormone. This result demonstrates that an altered binding profile can be achieved through loop grafting.

EXAMPLE 9 : Design of a prolactin framework

The CBD of human prolactin receptor has the following amino acid sequence:

```
24      GQLPPGK PEIFKCRSPN KETFTCWWRP GTDGGLPTNY
                           L1
61   SLTYHREGET LMHECPDYIT GGPNSCHFGK QYTSMWRTYI
        L2                          L3
101  MMVNATNQMG SSFSDELYVD VTYIVQPDPP LELAVEVKQP
                        L4
141  EDRKPYLWIK WSPPTLIDLK TGWFTLLYEI RLKPEKAAEW
                        L5
181  EIHFAGQQTE FKILSLHPGQ KYLVQVRCKP DHGYWSAWSP
           L6                       L7
221  ATFIQIPSD 229
```

The first FnIII-like domain is defined by amino acids Glu24 to Val125 and the second Fn-III like domain by Gln126 to Asp229. Loops L1 to L7 are indicated as boxed residues on the above sequence.

Modifications

A synthetic gene was designed on the basis of the amino acid listings above, expect with some modifications. In order to improve secretion, several changes were made to the gene construct. Lys30 was changed to Glu. Lysine or arginine charged residues within the first 10 amino acids at the N-terminus prevents the pelB secretion signal from working in the chosen expression system. Arg143Lys144 was changed to GlySer to remove the possibility of providing a proteolytic cleavage site and to provide a restriction enzyme site and a flexible replacement.

The gene was engineered to include convenient restriction sites for mutagensis and bacterial preferred codon usage for high level expression. In particular, the leucine and proline residues are changed.

In order to provide a scaffold library, any of the amino acids within any of the loops may be modified by using degenerate oligonucleotides to generate a diverse set of novel binding moieties as described in Example 7. In this case, the library will consist of a prolactin scaffold with a wide range of different amino acid loop compositions.

Single clones may be isolated from this library and their DNA sequenced to confirm the library diversity.

EXAMPLE 10: Design of a IL-11R scaffold.

The CBD of IL-11R has the following amino acid sequence:

```
111  YPPARPVVSC QAADYENFSC TWSPSQISGL PTRYLTSYRK
                     L1
151  KTVLGADSQR RSPSTGPWPC PQDPLGAARC VVHGAEFWSQ
       L2                                 L3
191  YRINVTEVNP LGASTRLLDV SLQSILRPDP PQGLRVESVP
                           L4
231  GYPRRLRASW TYPASWPCQP HFLLKFRLQY RPAQHPAWST
                        L5
271  VEPAGLEEVI TDAVAGLPHA VRVSARDFLD AGTWSTWSPE
       L6                          L7
321  AWGTPSTGT  329
```

The first FnIII-like domain is defined by amino acids 112-214 and the second FnIII-like domain by amino acids 218-318. Loops L1 to L7 are indicated as boxed residues on the above sequence.

Modifications.
In the IL-11R framework, the charged Arg115 may be replaced by Glu in order to improve expression in bacterial expression systems using secretion signals, e.g. PelB.

EXAMPLE 11: Multidomain scaffolds.

A scaffold consisting of the first FnIII-like domain derived from prolactin and the second FnIII-like domain derived from a human granulocyte colony stimulating factor receptor (G-CSFR) may be constructed.

The first FnIII-liek domain derived from the CBD of prolactin receptor is defined by residues 24-125 [IS THIS CORRECT - see Ex 9 questions]as in Example 9.

The CBD of GSCFR has the following amino acid sequence:

```
121  YPPAIPHNLS CLMNLTTSSL ICQWEPGPET HLPTSFTLKS
                        L1
161  FKSRGNCQTQ GDSILDCVPK DGQSHCCIPR KHLLLYQNMG
```

```
          L2                                  L3
201   IWVQAENALG TSMSPQLCLD PMD VVKLEPP ML RTMDPSPE
                              L4
241   AAPPQAGCLQ LCWEPWQPGL  HINQKCEL RH KP QRGEASWA
                                   L5
281   LVGPLP LEAL  Q YELCGLLPA TAYTLQIRC I RW PLP GHWSD
               L6                              L7
321   WSPSLELRTT ERA  333
```

Loops L1 to L7 of the CBD of GCSFR are indicated as boxed residues on the above sequence. The second region of the CBD of GCSFR is defined by residues 237-330.

Modifications.

In the G-CSFR framework there are several more cysteine residues in addition to the four conserved residues that form two disulphide bonds. Replacement of one or more of Cys186, Cys218, Cys248, Cys252 and Cys295 may therefore be necessary to provide expression of soluble proteins. In the first domain of the prolactin receptor, Lys30 can be changed to Glu as described above in Example 9.

A synthetic gene for the first domain of prolactin receptor and the second domain of GCSFR can be designed with convenient restriction sites and preferred codons as in previous examples. The gene can then be assembled into pHFAsacII phagemid vectors or ribosome display vectors. Phage can be produced and purified from bacterial cells transformed with phagemid using helper phage. Successful display of the scaffold can be confirmed by ELISA using specific targets.

Other modifications can also be made to the scaffold structure described herein, as will be evident to the skilled person. For example, loop L3 of the GCSFR can be extended (i.e. made longer) to form a 'protruding finger loop' by inserting extra amino acids. For example, an additional 5 residues can be inserted, either as a predetermined sequence (e.g. AYPPY) or as a random plurality of sequences encoded by a random mixture of 15-mer polynucleotides. Loop 3 can also be made shorter or deleted altogether to provide a possibly a smaller hinge area, and thereby provide a more restrained surface exposed scaffold. Similarly, any other loop in the CBD scaffold can be modified individually or collectively using similar designs to loop 4 as described above.

Other potential modifications include inserting amino acids in areas not specifically associated with the loop region. such as in the hinge region or the domain interface.

EXAMPLE 12: Multivalent and Multispecific Scaffolds.

It is possible to form multivalent and multispecific scaffolds by either genetic or chemical linkage of two modified cytokine binding domains of the invention. Both linkage formats can result in either covalent or non-covalent bonds or a combination of covalent and non-covalent bonds to effect the association of two or more cytokine binding domains. It will be evident to the skilled person that single cytokine binding domains, or the multivalent or multispecific formats can be genetically or chemically linked to plurality of molecules or linked to a variety of surfaces.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are readily apparent to those skilled in molecular biology or related fields are intended to be within the scope of the invention.

CLAIMS

1. A method of producing a binding moiety comprising
modifying an extracellular cytokine binding domain consisting of a first FnIII-like domain and a second FnIII-like domain such that at least one property of the cytokine binding domain is altered,
to produce a binding moiety.

2. The method according to claim 1, wherein the first and second FnIII-like domains are derived from the extracellular cytokine binding domain from a single source.

3. The method according to claim 1, wherein the first and second FnIII-like domains are derived from the extracellular cytokine binding domains from separate sources.

4. The method according to any preceding claim, wherein the first and/or second FnIII-like domain(s) is/are derived from the extracellular domain of a receptor selected from IL-2 receptor, IL-3 receptor, IL-4 receptor, IL-5 receptor, IL-6 receptor, IL-7 receptor, IL-9 receptor, IL-11 receptor, IL-12 receptor, IL-13 receptor, IL-15 receptor and IL-21 receptor, G-CSF receptor, GM-CSF receptor, LIF receptor, oncostatin M receptor, cardiotrophin CT-1 receptor, ciliary neutrotrophic factor (CNTF) receptor, prolactin receptor, leptin receptor, erythropoietin receptor, growth hormone receptor, cytokine receptor-like factor 1, class 1 cytokine receptor, thymic stromal lymphopoietin protein receptor or gp130.

5. The method according to any preceding claim, wherein at least one loop of the cytokine binding domain is modified.

6. The method according to claim 5, wherein the loops are defined by loops L1 to L7 as indicated in Figure 5.

7. The method according to claim 5 or claim 6, wherein the size and/or area of the loop is modified as compared with the corresponding loop in the unmodified cytokine binding domain.

8. The method according to claim 7, wherein the size of the loop is increased or reduced by at least two amino acid residues.

9. The method according to claim 8, wherein the size of the loop is increased by at least 10 amino acid residues

10. The method according to claim 8 or claim 9, wherein the size of the loop is increased by up to 20 amino acid residues.

11. The method according to any preceding claim, wherein the hinge region between the two FnIII-like domains is modified.

12. The method according to any preceding claim, wherein the binding interface of the FnIII-like domains of the cytokine binding domain is modified.

13. The method according to any preceding claim, wherein one or more intra-domain disulphide-bond forming cysteine residues in the cytokine binding domain are modified.

14. The method according to any preceding claim, wherein the solubility of the binding moiety is improved.

15. The method according to claim 14, wherein the solubility of the binding moiety is improved by removing and/or replacing disulphide-bond forming cysteine residues within in the cytokine binding domain.

16. The method according to any one of claims 13 to 15, wherein disulphide-bond forming cysteine residues are replaced by a different residue.

17. The method according to claim 16, wherein disulphide-bond forming cysteine residues are replaced by alanine or serine.

18. The method according to any preceding claim, wherein the stability of the cytokine binding domain is improved.

19. The method according to any preceding claim, wherein the affinity of the modified cytokine binding domain for at least one natural ligand of the unmodified cytokine binding domain is reduced or abolished.

20. The method according to any preceding claim, wherein the binding specificity of the modified cytokine binding domain is different to that of the unmodified cytokine binding domain.

21. The method according to claim 20, wherein the unmodified cytokine binding domain is derived from the extracellular domain of a first receptor having specificity for a first ligand, and the modification comprises replacing one or more loops of the unmodified cytokine binding domain with the corresponding loops of a second receptor having specificity for a second ligand such that the modified cytokine binding domain has specificity for the second ligand.

22. The method according to claim 21, wherein the first receptor is IL6 receptor and the second receptor is prolactin receptor or LIF receptor.

23. The method according to claim 22, wherein the first receptor is IL6 receptor and the second receptor is oncostatin M receptor.

24. The method according to any preceding claim, further comprising linking the modified binding moiety to one or more molecules.

25. The method according to claim 24, wherein the modified binding moiety is linked to one or more molecules via a genetic or chemical linker.

26. The method according to claim 24 or claim 25, wherein the modified binding moiety is linked to one or more molecules via a covalent or non-covalent linkage.

27. The method according to any one of claims 24 to 26, wherein the modified binding moiety is linked to a diagnostic reagent or a therapeutic agent.

28. The method according to claim 27, wherein the diagnostic reagent is a detectable label.

29. The method according to claim 27, wherein the therapeutic agent is cytotoxic.

30. The method according to claim 27, wherein the therapeutic agent is immunomodulatory.

31. A modified binding moiety produced by any one of the methods according to claims 1 to 30.

32. A binding moiety comprising an extracellular cytokine binding domain consisting of a first FnIII-like domain and a second FnIII-like domain, wherein the cytokine binding domain comprises a modification which alters at least one property of the cytokine binding domain.

33. The binding moiety according to claim 32, wherein the first and second FnIII-like domains are derived from the extracellular cytokine binding domain from a single source.

34. The binding moiety according to claim 33, wherein the first and second FnIII-like domains are derived from the extracellular cytokine binding domains from separate sources.

35. The binding moiety according to any one of claims 32 to 34, wherein the first and/or second FnIII-like domain(s) is/are derived from the extracellular domain of a receptor selected from IL-2 receptor, IL-3 receptor, IL-4 receptor, IL-5 receptor, IL-6 receptor, IL-7 receptor, IL-9 receptor, IL-11 receptor, IL-12 receptor, IL-13 receptor, IL-15 receptor and IL-21 receptor, G-CSF receptor, GM-CSF receptor, LIF receptor, oncostatin M receptor, cardiotrophin CT-1 receptor, ciliary neutrotrophic factor (CNTF) receptor, prolactin receptor, leptin receptor, erythropoietin receptor, growth hormone receptor, cytokine receptor-like factor 1, class 1 cytokine receptor, thymic stromal lymphopoietin protein receptor or gp130.

36. The binding moiety according to any one of claims 32 to 35, wherein a loop of the cytokine binding domain is modified.

37. The binding moiety according to claim 36, wherein the loops are defined by loops L1 to L7 as indicated in Figure 5.

38. The binding moiety according to claim 36 or claim 37, wherein the size and/or area of the loop is modified as compared with the corresponding loop in the unmodified cytokine binding domain.

39. The binding moiety according to claim 38, wherein the size of the loop is increased or reduced by at least two amino acid residues.

40. The binding moiety according to claim 39, wherein the size of the loop is increased by at least 10 amino acid residues.

41. The binding moiety according to claim 39 or claim 40, wherein the size of the loop is increased by up to 20 amino acid residues.

42. The binding moiety according to any one of claims 32 to 41, wherein the hinge region between the FnIII-like domains is modified.

43. The binding moiety according to any one of claims 32 to 42, wherein the binding interface of the FnIII-like domains is modified.

44. The binding moiety according to any one of claims 32 to 43, wherein one or more of intra-domain disulphide-bond forming cysteine residues in the cytokine binding domain is modified

45. The binding moiety according to any one of claims 32 to 44, wherein the solubility of modified binding moiety is improved.

46. The binding moiety according to claim 45, wherein the solubility of the binding moiety is improved by removing and/or replacing disulphide-bond forming cysteine residues within the cytokine binding domain.

47. The binding moiety according to any one of claims 44 to 46, wherein disulphide-bond forming cysteine residues are replaced by a different residue.

48. The binding moiety according to claim 47, wherein disulphide-bond forming cysteine residues are replaced with alanine or serine.

49. The binding moiety according to any one of claims 32 to 48, wherein the stability of the cytokine binding domain is improved.

50. The binding moiety according to any one of claims 32 to 47, wherein the affinity of the modified cytokine binding domain for at least one natural ligand of the unmodified cytokine binding domain is reduced or abolished

51. The binding moiety according to any one of claims 32 to 50, wherein the binding specificity of the modified cytokine binding domain is different to that of the unmodified cytokine binding domain.

52. The binding moiety according to claim 51, wherein the unmodified cytokine binding domain is derived from the extracellular domain of a first receptor having specificity for a first ligand, one or more loops of the unmodified cytokine binding domain have been replaced with the corresponding loops of a second receptor having specificity for a second ligand, and the modified cytokine binding domain has specificity for the second ligand.

53. The binding moiety according to claim 52, wherein the first receptor is IL-6 receptor and the second receptor is prolactin receptor or LIF receptor.

54. The binding moiety according to claim 52, wherein the first receptor is IL-6 receptor and the second receptor is oncostatin M receptor.

55. The binding moiety according to any one of claims 31 to 54 linked to one or more molecules.

56. The binding moiety according to claim 55, linked to one or more molecules via a genetic or chemical linker.

57. The binding moiety according to 55 or claim 56, linked to one or more molecules via a covalent or non-covalent linkage.

58. The binding moiety according to any one of claims 55 to 57, linked to a diagnostic reagent or a therapeutic agent.

59. The binding moiety according to claim 58, wherein the diagnostic reagent is a detectable label.

60. The binding moiety according to claim 58, wherein the therapeutic agent is cytotoxic.

61. The binding moiety according to claim 58, wherein the therapeutic agent is immunomodulatory.

62. A multivalent or multispecific reagent comprising two or more binding moieties according to any one of claims 31 to 61.

63. The binding moiety, multivalent reagent or multispecific reagent according to any one of claims 31 to 61, immobilised on a solid support or coupled to a biosensor surface.

64. A polynucleotide encoding a binding moiety, multivalent reagent or multispecific reagent according to one of claims 31 to 62.

65. A vector comprising a polynucleotide according to claim 64.

66. A host cell comprising a vector according to claim 65.

67. A pharmaceutical composition comprising a binding moiety, multivalent reagent or multispecific reagent according to any one of claims 31 to 62 and a pharmaceutically acceptable carrier or diluent.

68. A method of treating a pathological condition in a subject, which method comprises administering to the subject a binding moiety, multivalent reagent or multispecific reagent according to any one of claims 31 to 62.

69. A method of selecting a binding moiety with an affinity for a target molecule which comprises

(i) providing a plurality of polynucleotides encoding binding moieties comprising a cytokine binding domain, which polynucleotides comprise one or more modifications in the cytokine biding domain;

(ii) expressing the binding moieties encoded by the polynucleotides; and

(iii) selecting one or more binding moieties having an affinity for the target molecule.

70. The method according to claim 69, wherein the modification(s) is/are in the loop(s) of the cytokine binding domain.

71. The method according to claim 69 or claim 70, wherein the plurality of nucleotides have been subjected to mutagenesis.

72. The method according to claim 71, wherein the mutagenesis is site-directed mutagenesis.

73. The method according to claim 72, wherein the mutagenesis is random mutagenesis.

74. A method according to any one of claims 69 to 72, wherein the target molecule is a cytokine receptor ligand.

75. A polynucleotide library comprising a plurality of polynucleotides encoding binding moieties comprising a cytokine binding domain, which polynucleotides comprise one or more modifications in the cytokine biding domain.

76. A nucleic acid sequence encoding:
a) a first scaffold sequence encoding a cytokine binding domain; and
b) a second sequence encoding a peptide and inserted at a site located in a region of said first scaffold sequence, said peptide being displayed as a loop.

77. The nucleic acid sequence according to claim 76, wherein the second sequence substantially replaces the region of the first scaffold sequence encoding the loop.

78. The nucleic acid sequence according to claim 76 or claim 77, wherein the cytokine binding domain is derived from the extracellular domain of a receptor selected from IL-2 receptor, IL-3 receptor, IL-4 receptor, IL-5 receptor, IL-6 receptor, IL-7 receptor, IL-9 receptor, IL-11 receptor, IL-12 receptor, IL-13 receptor, IL-15 receptor and IL-21 receptor, G-CSF receptor, GM-CSF receptor, LIF receptor, oncostatin M receptor, cardiotrophin CT-1 receptor, ciliary neutrotrophic factor (CNTF) receptor, prolactin receptor, leptin receptor, erythropoietin receptor, growth hormone receptor, cytokine receptor-like factor 1, class 1 cytokine receptor, thymic stromal lymphopoietin protein receptor or gp130.

79. The nucleic acid sequence according to any one of claims 76 to 78, comprising a plurality of second sequences inserted into a plurality of sites.

80. The nucleic acid sequence according to any one of claims 76 to 79, wherein one or more of the peptides are derived from a different cytokine binding region to that of the scaffold sequence.

81. An expression vector comprising a nucleic acid sequence according to any one of claims 76 to 80.

82. A cytokine binding domain display library comprising a plurality of expression vectors according to claim 81.

83. An expression vector comprising:
a) a first nucleic acid sequence encoding a cytokine binding domain;
b) an insertion site in a region between the ends of the first nucleic acid sequence, the insertion site comprising a nucleotide sequence which is cleaved by a restriction endonuclease and which allows a second nucleic acid sequence encoding an amino acid sequence to be inserted into the first nucleic acid to encode a modified cytokine binding domain; and
c) a regulatory control sequence operably linked to said first nucleic acid sequence which directs expression of the first nucleic acid sequence.

84. An expression vector comprising:
a) a first nucleic acid sequence encoding a cytokine binding domain, said sequence comprising a deletion in a region between the ends of the first nucleic acid sequence;
b) an insertion site in place of the deleted sequence which site allows a second nucleic acid sequence encoding an amino acid sequence to be inserted into the first nucleic acid to encode a modified cytokine binding domain.
c) a regulatory control sequence operably linked to said first nucleic acid sequence which directs expression of the first nucleic acid sequence.

85. The expression vector according to claim 83 or claim 84, wherein the region in which the insertion site or deletion is present encodes a loop.

86. A polypeptide encoded by the nucleic acid sequence of any one of claims 76 to 80.

87. A protein multimer comprising at least two polypeptides according to claim 86.

88. A method of identifying a modified cytokine binding domain which binds to a target molecule of interest, which method comprises:
(i) providing a cytokine binding domain display library according to claim 82;
(ii) expressing the polypeptides encoded by the polynucleotides; and
(iii) selecting one or more polypeptides that bind to the target molecule.

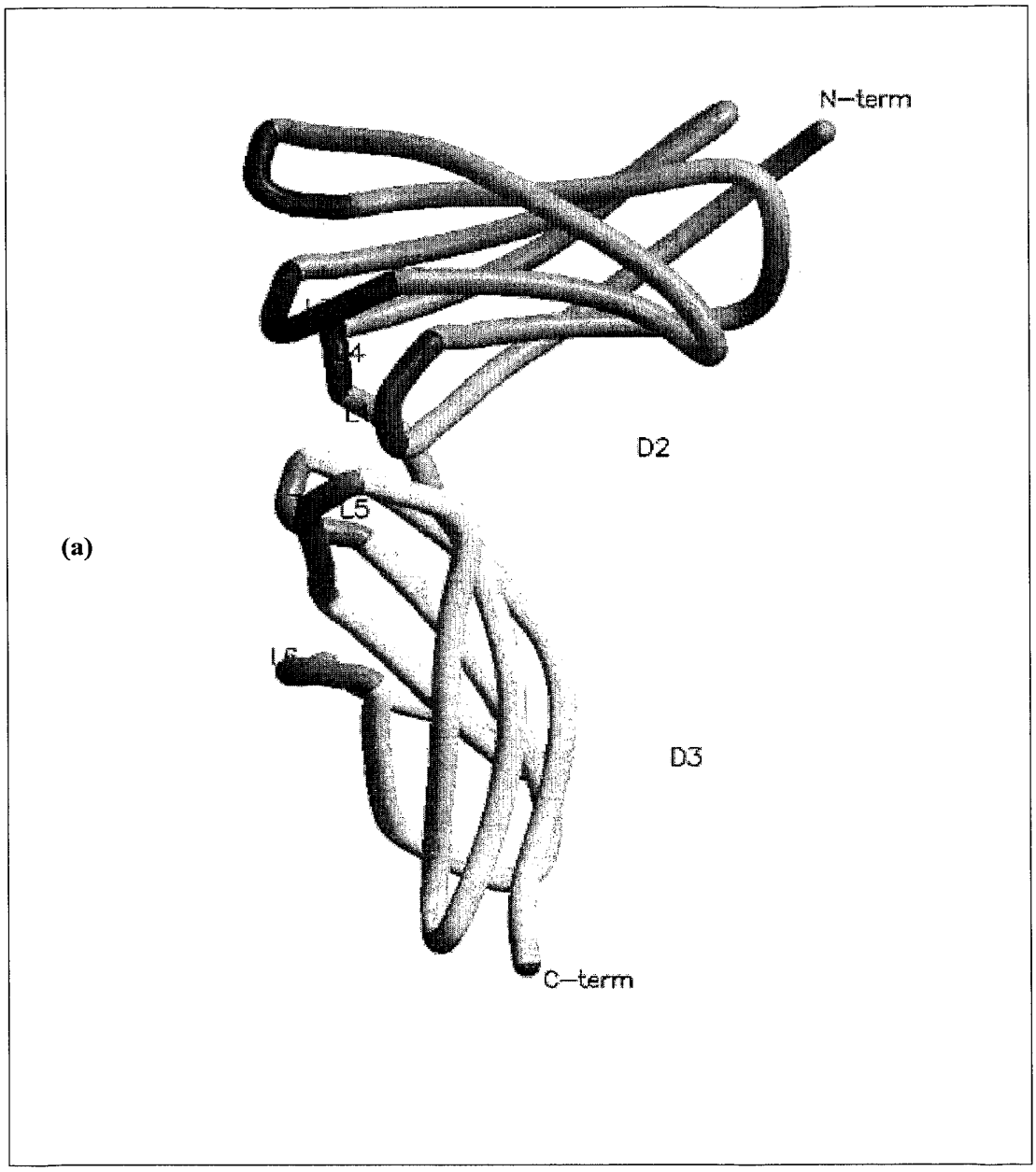


Figure 1(a)

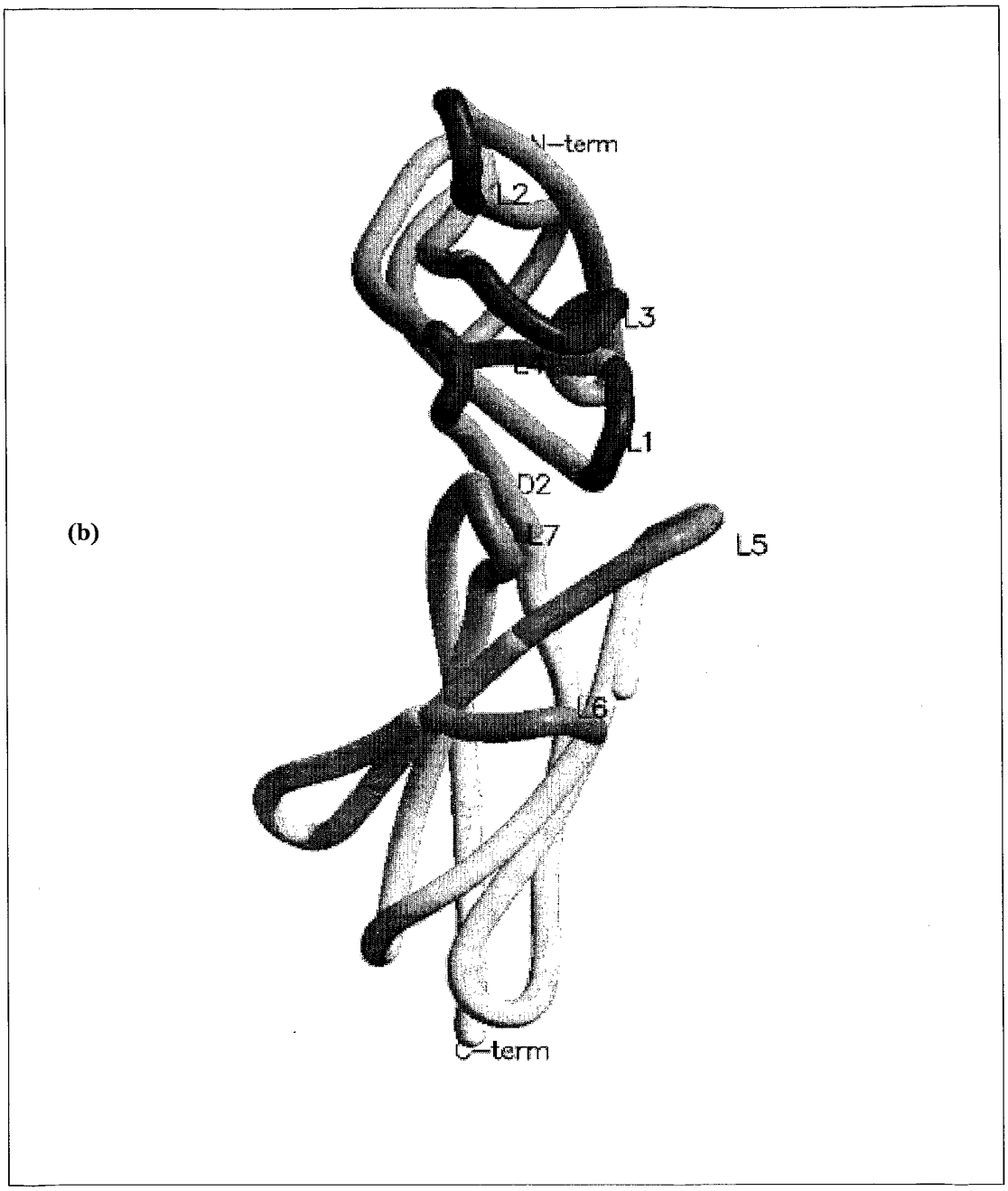


Figure 1(b)

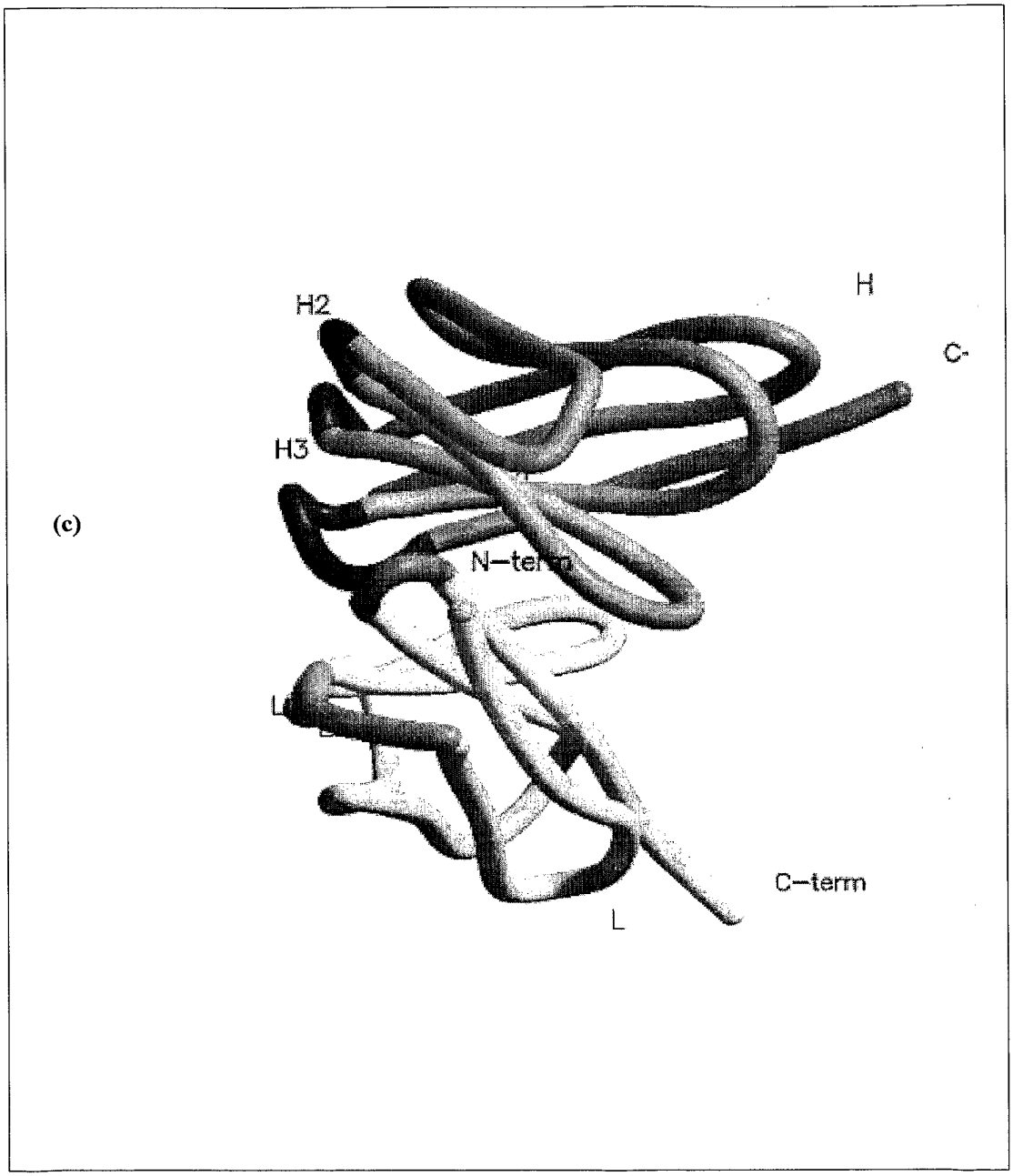


Figure 1(c)

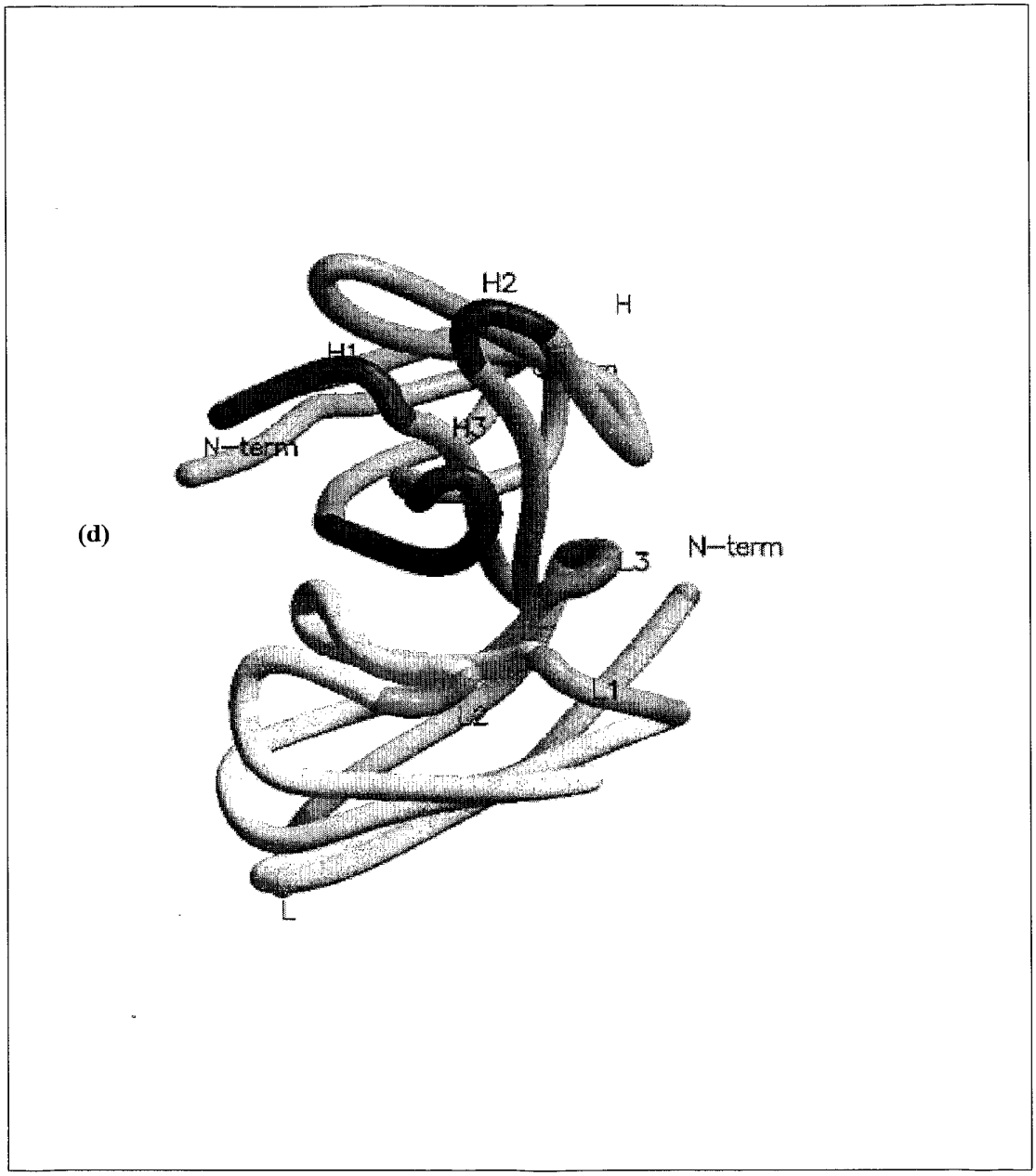


Figure 1(d)

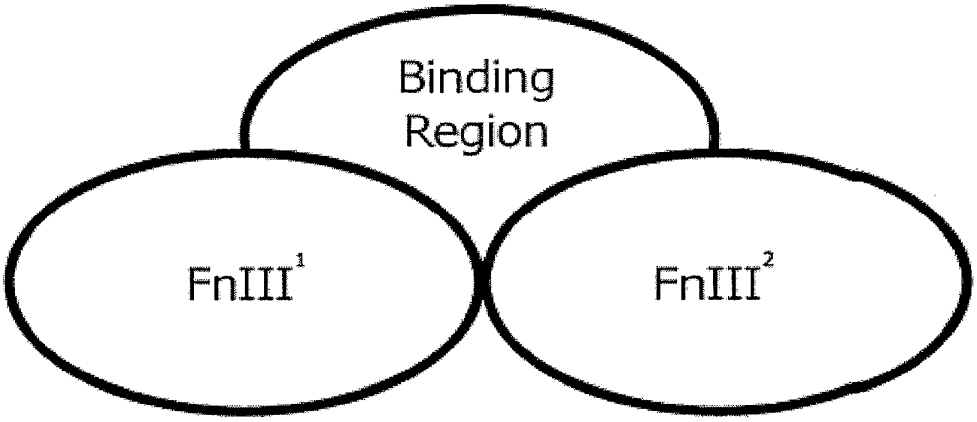


Figure 1A

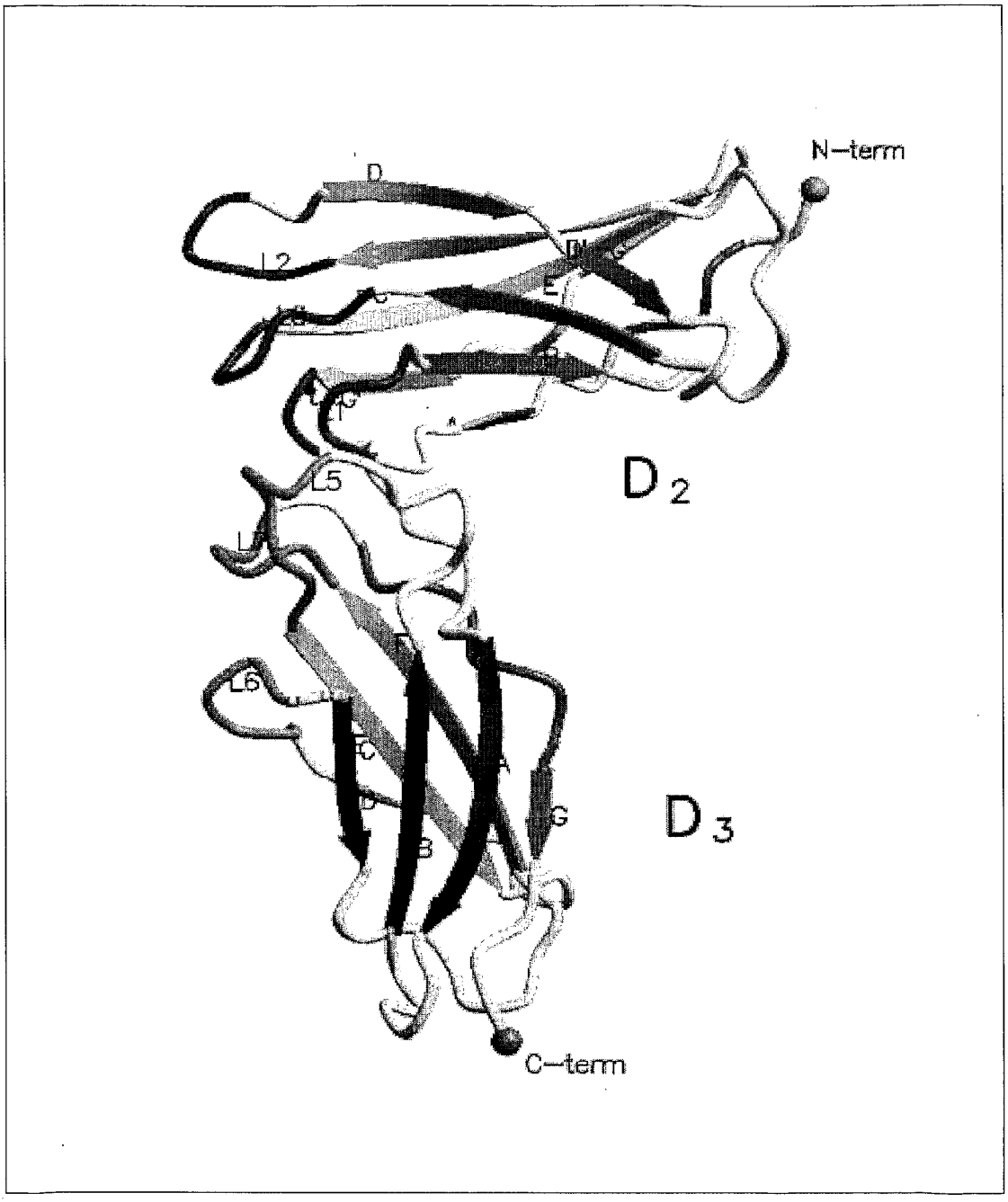


Figure 2(a)

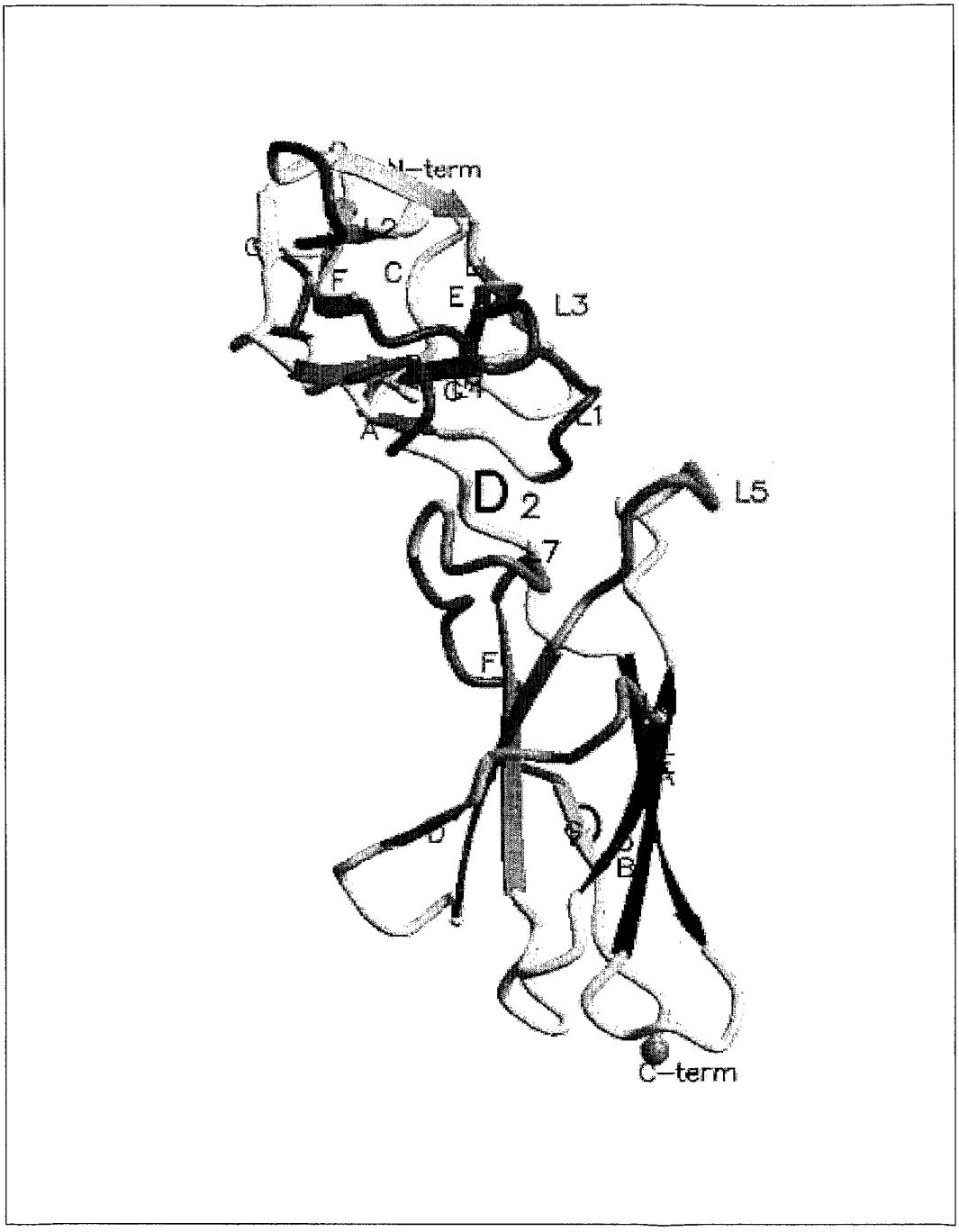
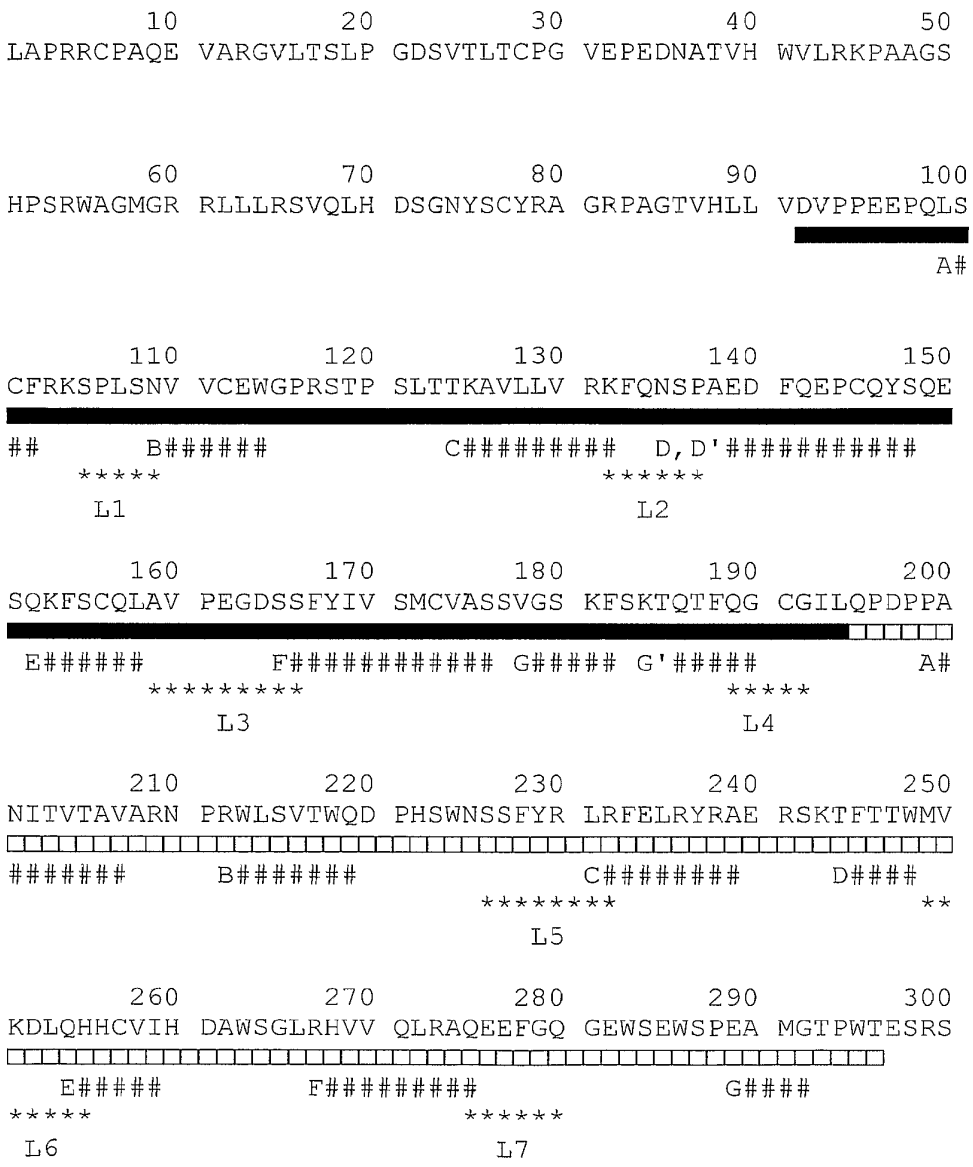


Figure 2(b)



beta sheets; * loops; ■ first domain (D2); □ second domain (D3)

Figure 3

Approximate positioning of each loop in four of the cytokine receptor family members. The loop positions could vary up to 3 amino acids either side of the box. For example Loop 6 of the prolactin receptor is defined as GQQTEF and not FAQQ as depicted here.

```
IL6RPRLRL          ------------------------ VPPEEPQ-LSCFSPNK-ETFVCEWGPRSTPSLTTK
P08887|IL6A_HUMAN  LHDSGNYSCY-RAGRPAGTVHLLV VPPEEPQ-LSCFRKSPLSNVVCEWGPRSTPSLTTK
Q14626|I11R_HUMAN  STDEGTYICQTLDGALGGTVTLQL YPPARPV-VSCQAADY-ENFSCTWSPSQISGLPTR
P16471|PRLR_HUMAN  MKENVASATVFTLLLFLNTCLLNG LPPGKPEIFKCRSPNK-ETFTCWWRPGTDGGLPTN
Q99062|GCSR_HUMAN  -AFLSCCLNWGNSLQILDQVELRA YPPAIPHNLSCLMNLTTSSLICQWEPGPETHLPTS
                                                          L1

IL6RPRLRL          AVLLVHRE---------GETLMFQEPCQYSQESQKFSCHFGKQYTSMWRTYIVSMSVASS
P08887|IL6A_HUMAN  AVLLVRKFQN-------SPAEDFQEPCQYSQESQKFSCQLAVPEGD-SSFYIVSMCVASS
Q14626|I11R_HUMAN  YLTSYRKKTVLGADSQRRSPSTGPWPCPQD-PLGAARCVVHGAEFW--SQYRINVTEVNP
P16471|PRLR_HUMAN  YSLTYHRE-----------GETLMHECPDYITGGPNSCHFGKQYTSMWRTYIMMVNATNQ
Q99062|GCSR_HUMAN  FTLKSFKSRNC------QTQGDSILDCVPK-DGQSHCCIPRKHLLLYQNMGIWVQAENAL
                           L2                                  L3

IL6RPRLRL          VGSKFSDELYVDVTYILQPDPPANITVTAVA-RNPR---WLSVTWQDPHLIDLK-TGWFT
P08887|IL6A_HUMAN  VGSKFSKTQTFQGCGILQPDPPANITVTAVA-RNPR---WLSVTWQDPHSWNSS---FYR
Q14626|I11R_HUMAN  -LGASTRLLDVSLQSILRPDPPQGLRVESVP-GYPR---RLRASWTYPASWPCQ--PHFL
P16471|PRLR_HUMAN  MGSSFSDELYVDVTYIVQPDPPLELAVEVKQ-PEDR-KPYLWIKWSPPTLIDLK-TGWFT
Q99062|GCSR_HUMAN  GTSMSPQTLCLDPMDVVKLEPPMLRTMDPSPEAAPPQAGCLQLCWEPWQPGLHINQKCEL
                           L4                                                L5

IL6RPRLRL          LRFELRYRAERSKTFTTWFAG-QQHHSVIHDAWSGLRHVVQLRAKPD--HGYWSEWSPEA
P08887|IL6A_HUMAN  LRFELRYRAERSKTFTTWMVKDLQHHCVIHDAWSGLRHVVQLRAQEEFGQGEWSEWSPEA
Q14626|I11R_HUMAN  LKFRLQYRPAQHPAWSTVEPAG--LEEVITDAVAGLPHAVRVSARDFLDAGTWSTWSPEA
P16471|PRLR_HUMAN  LLYEIRLKPEKAAEWEIHFAGQ-QTEFKILSLHPGQKYLVQVRCKPD--HGYWSAWSPAT
Q99062|GCSR_HUMAN  RHKPQRGEASWALVGPLPLEAL-QYELCGLLP--ATAYTLQIRCIRWPLPGHWSDWSPSL
                                 L6                          L7

IL6RPRLRL          MGTPWTE ----------------------------------------------------
P08887|IL6A_HUMAN  MGTPWTE RSPPAENEVST----------PMQALTTN---KDDDNILFRDSANATSLPVQ
Q14626|I11R_HUMAN  WGTPSTG IPKEIPAWGQL----------HTQPEVEP---QVDSPAPPRPSLQPHPRLLD
P16471|PRLR_HUMAN  FIQIPSD TMNDTTVWISVAVLSAVICLIIVWAVALKGYSMVTCIFPPVPGPKIKGFDAH
Q99062|GCSR_HUMAN  ELRTTER PTVRLDTWWRQR-QLDPRTVQLFWKPVPLEEDSGRIQGYVVS-WRPSGQAGA
```

Figure 3A

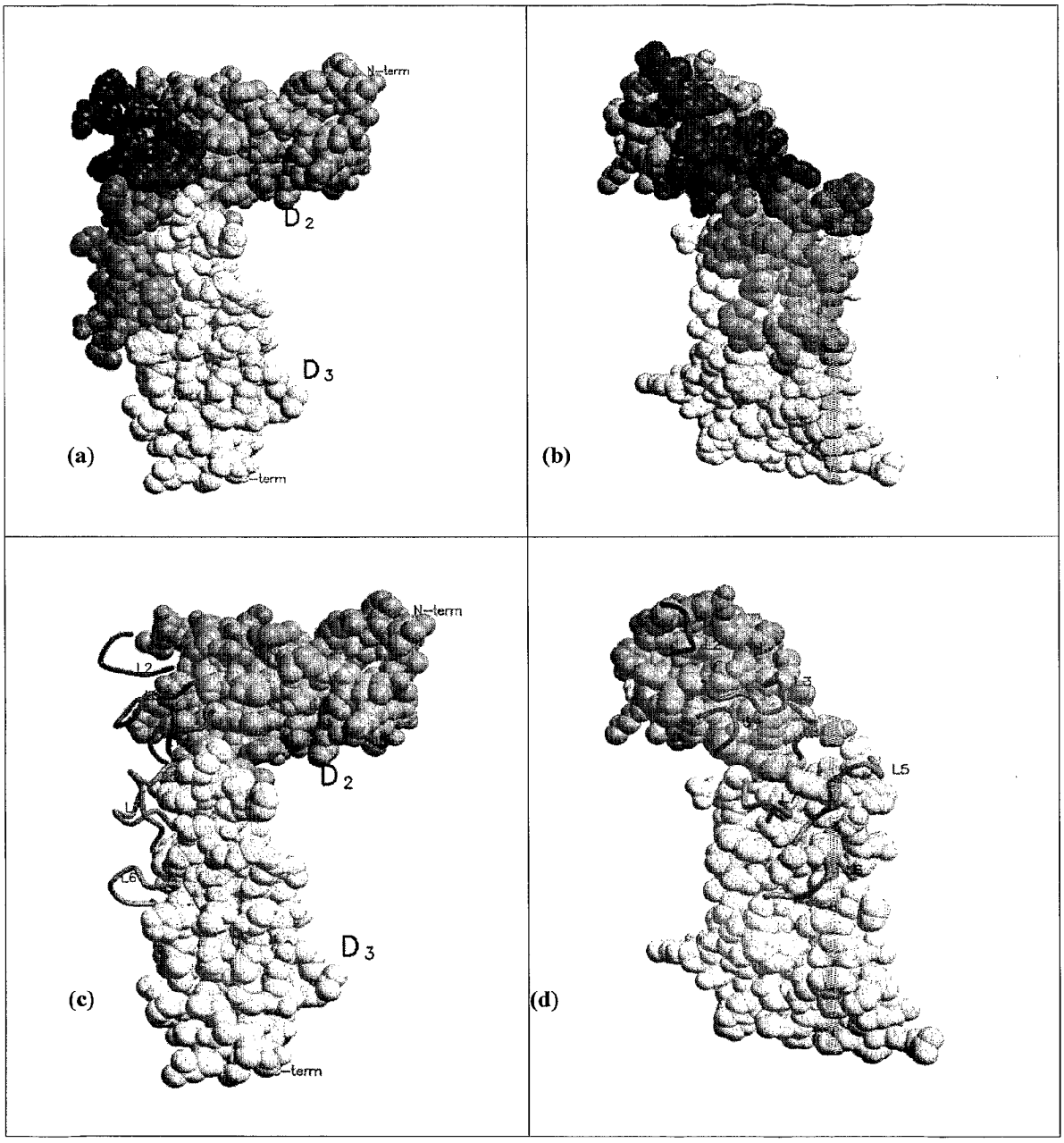


Figure 4

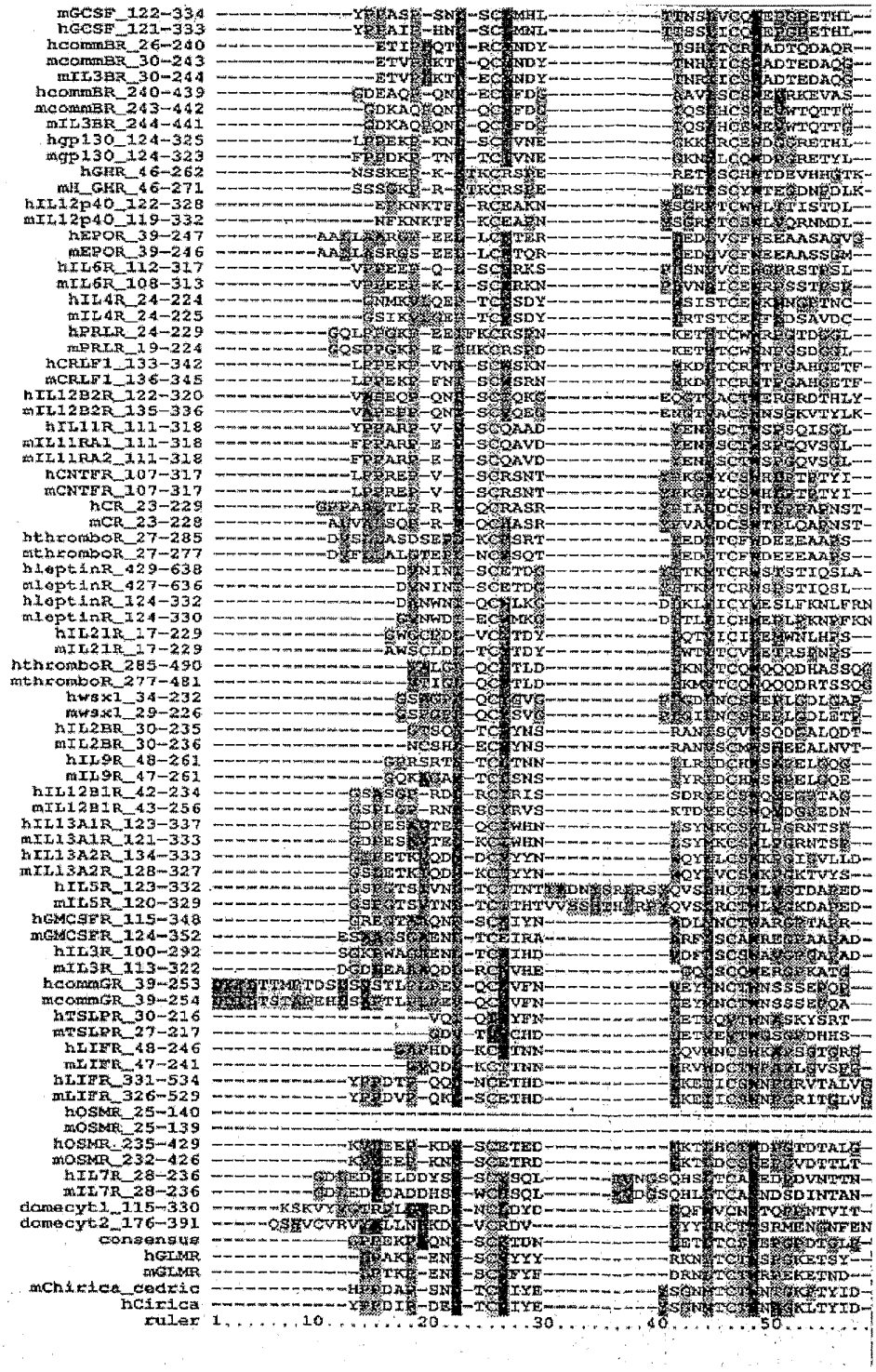

mGCSF_122-334
hGCSF_121-333
hcommBR_26-240
mcommBR_30-243
mIL3BR_30-244
hcommBR_240-439
mcommBR_243-442
mIL3BR_244-441
hgp130_124-325
mgp130_124-323
hGHR_46-262
mH_GHR_46-271
hIL12p40_122-328
mIL12p40_119-332
hEPOR_39-247
mEPOR_39-246
hIL6R_112-317
mIL6R_108-313
hIL4R_24-224
mIL4R_24-225
hPRLR_24-229
mPRLR_19-224
hCRLF1_133-342
mCRLF1_136-345
hIL12B2R_122-320
mIL12B2R_135-336
hIL11R_111-318
mIL11RA1_111-318
mIL11RA2_111-318
hCNTFR_107-317
mCNTFR_107-317
hCR_23-229
mCR_23-228
hthromboR_27-285
mthromboR_27-277
hleptinR_429-638
mleptinR_427-636
hleptinR_124-332
mleptinR_124-330
hIL21R_17-229
mIL21R_17-229
hthromboR_285-490
mthromboR_277-481
hwsx1_34-232
mwsx1_29-226
hIL2BR_30-235
mIL2BR_30-236
hIL9R_48-261
mIL9R_47-261
hIL12B1R_42-234
mIL12B1R_43-256
hIL13A1R_123-337
mIL13A1R_121-333
hIL13A2R_134-333
mIL13A2R_128-327
hIL5R_123-332
mIL5R_120-329
hGMCSFR_115-348
mGMCSFR_124-352
hIL3R_100-292
mIL3R_113-322
hcommGR_39-253
mcommGR_39-254
hTSLPR_30-216
mTSLPR_27-217
hLIFR_48-246
mLIFR_47-241
hLIFR_331-534
mLIFR_326-529
hOSMR_25-140
mOSMR_25-139
hOSMR_235-429
mOSMR_232-426
hIL7R_28-236
mIL7R_28-236
domecyt1_115-330
domecyt2_176-391
consensus
hGLMR
mGLMR
mChirica_cedric
hCirica
ruler 1.....10.....20.....30.....40.....50

Figure 5A

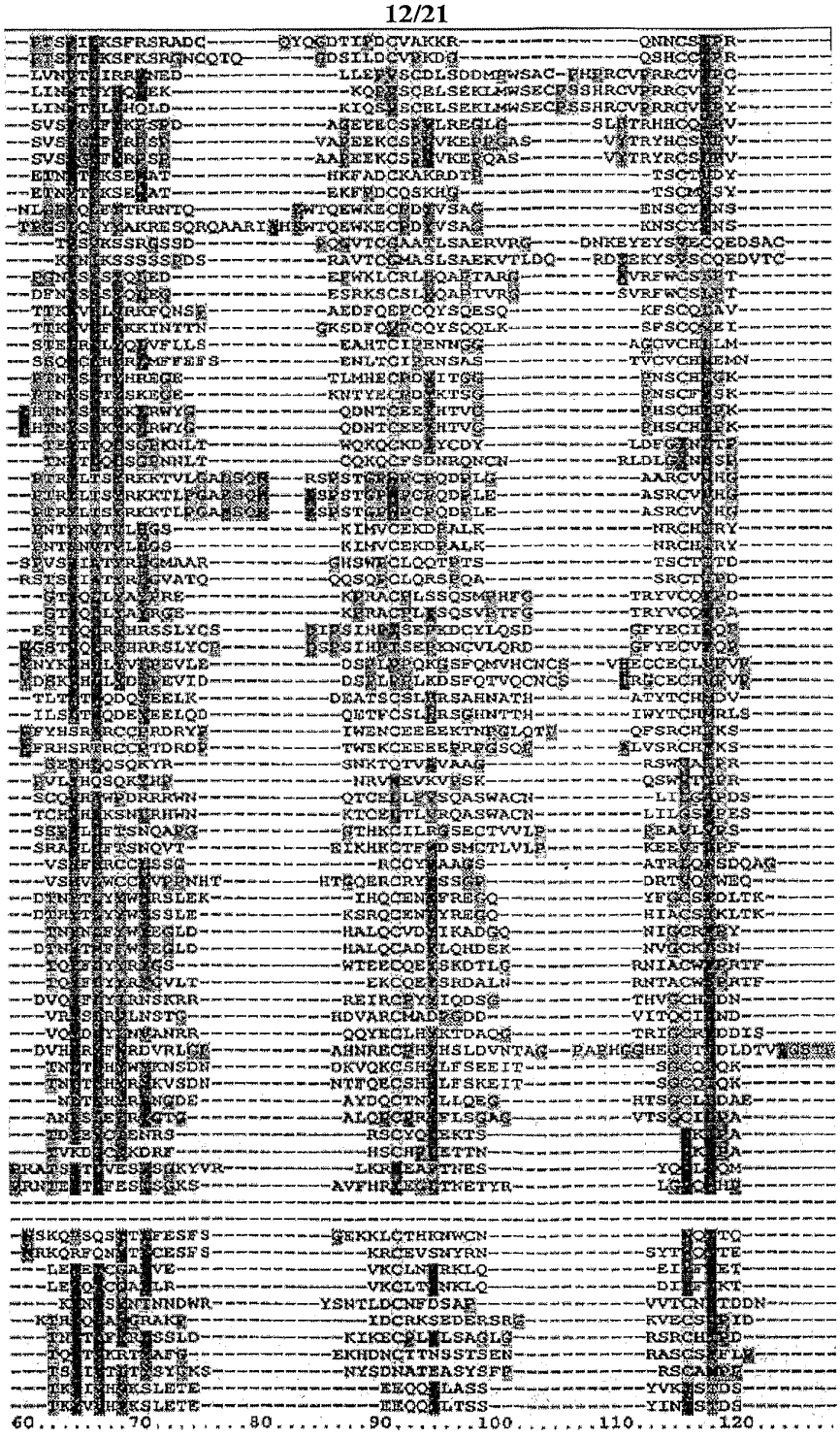


Figure 5A (cont)

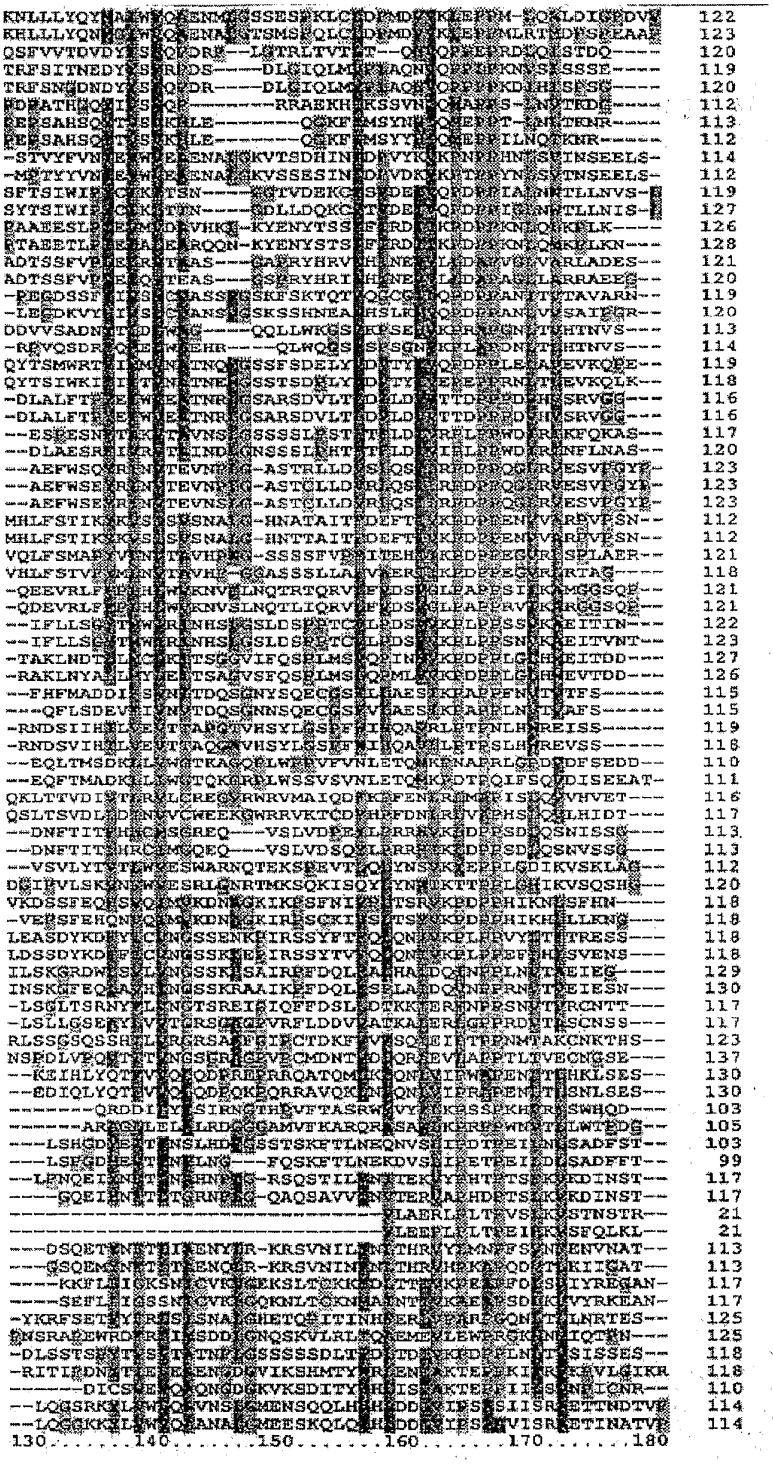


Figure 5A (cont)

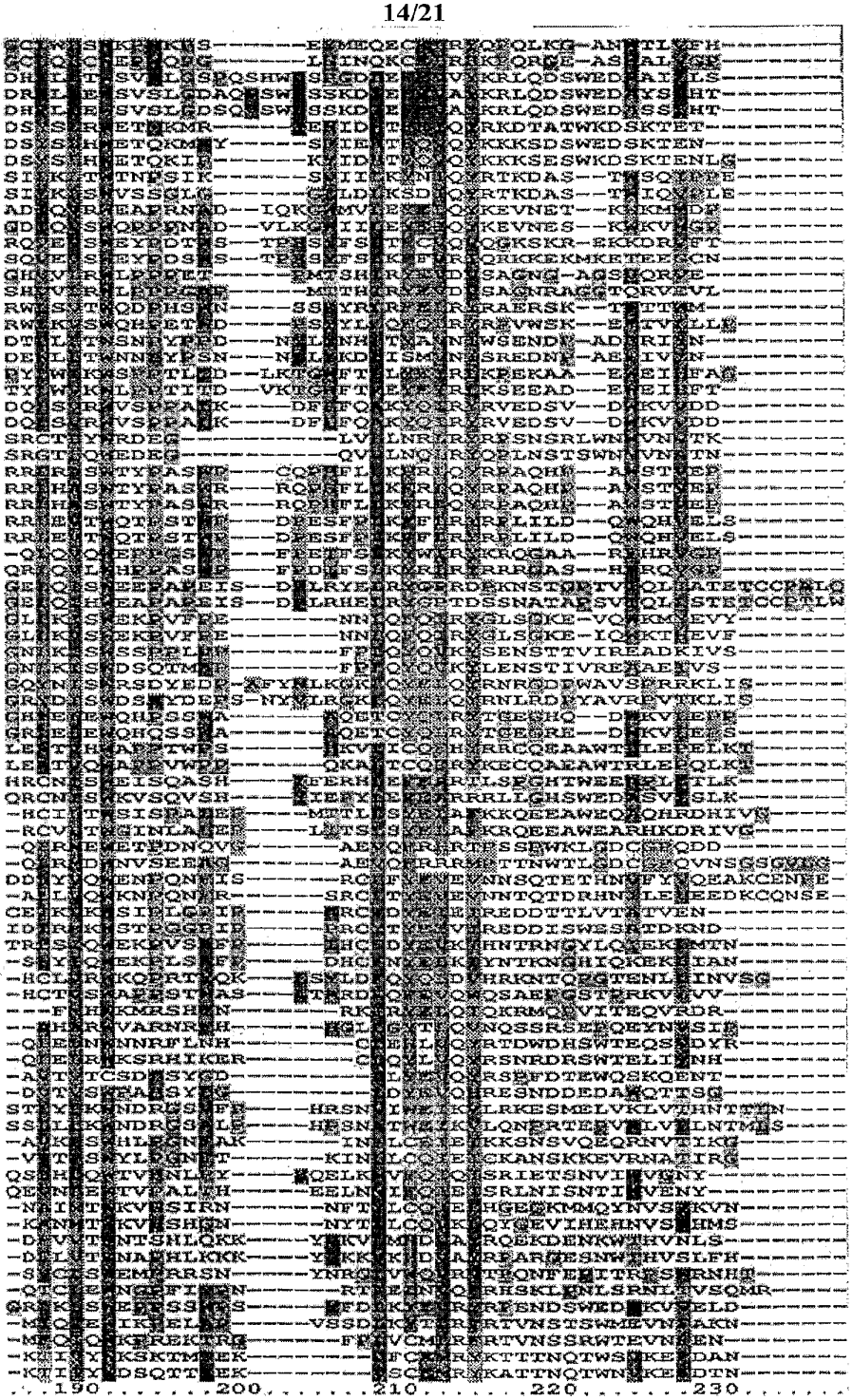


Figure 5B

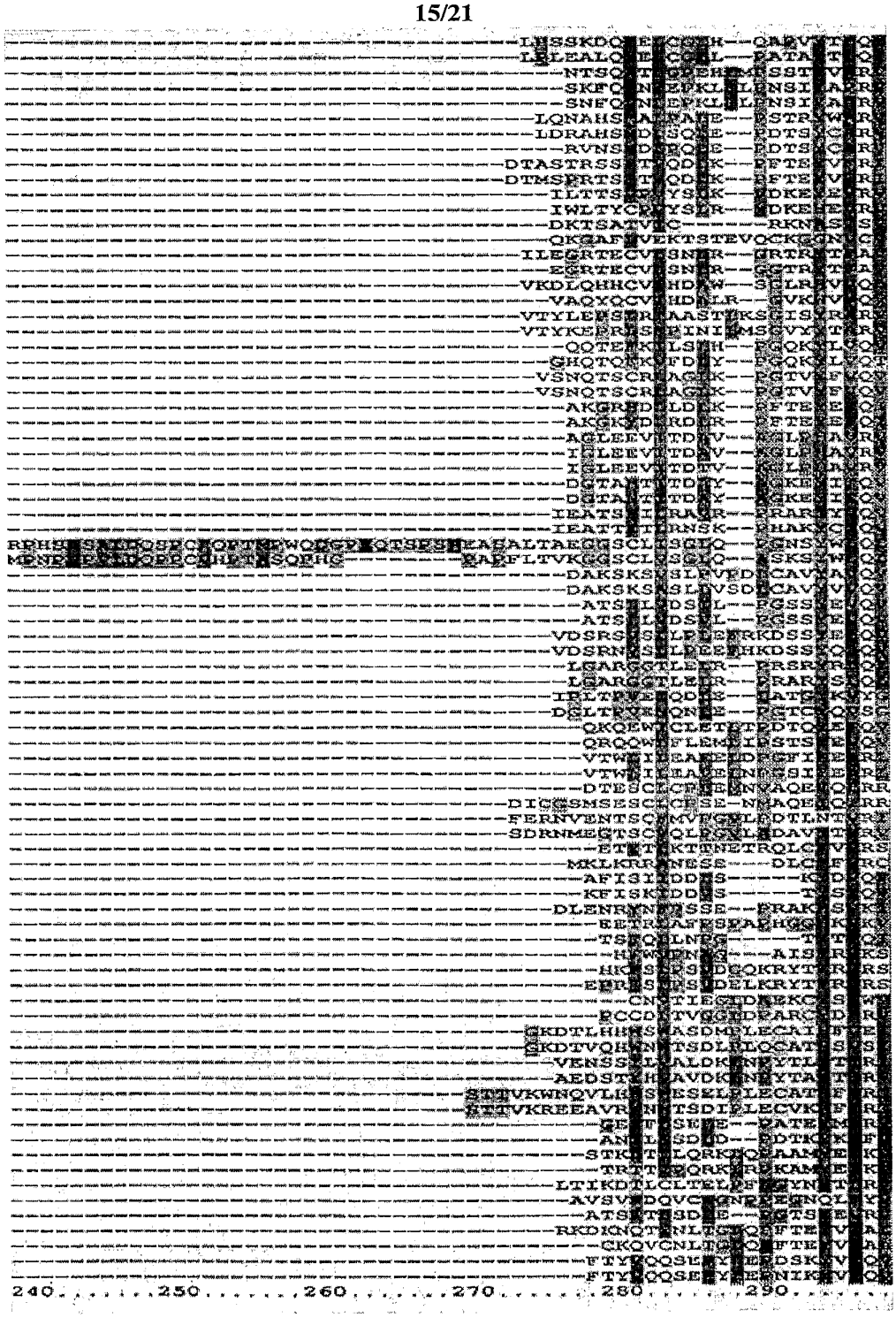


Figure 5B (cont)

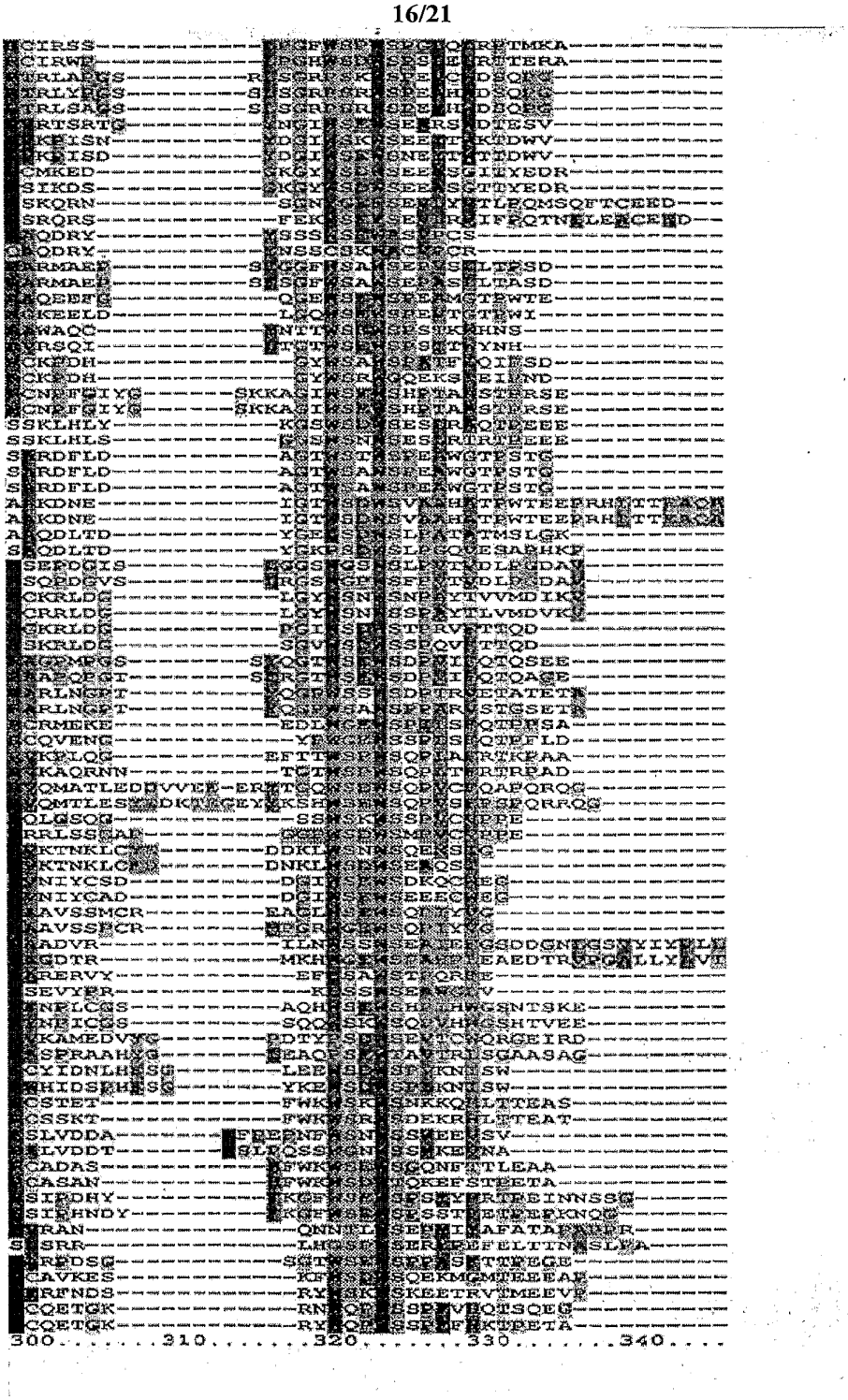


Figure 5B (cont)

(a)

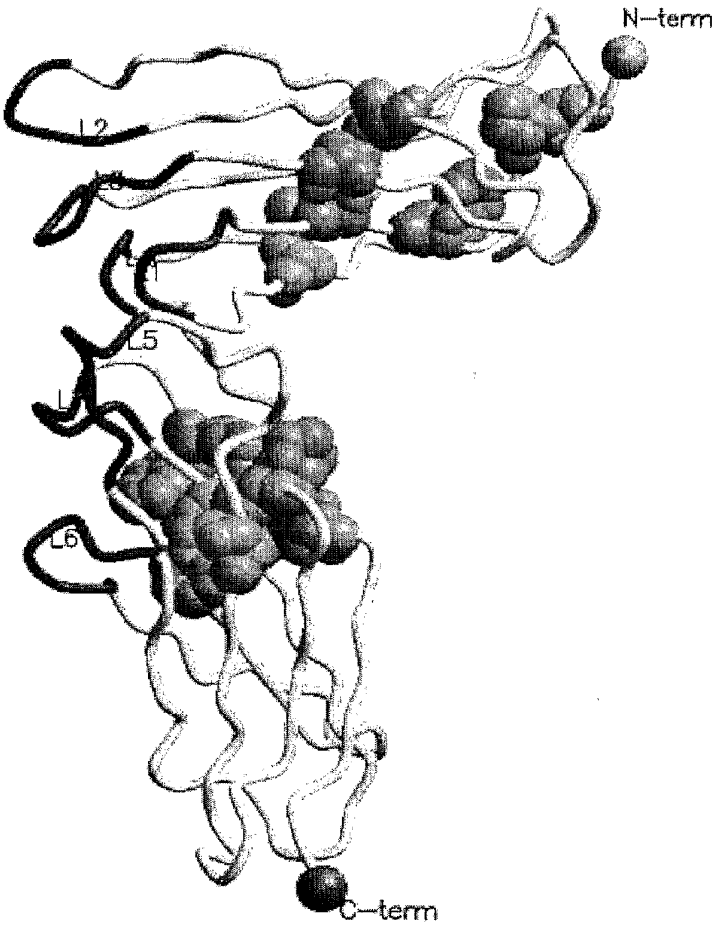


Figure 6(a)

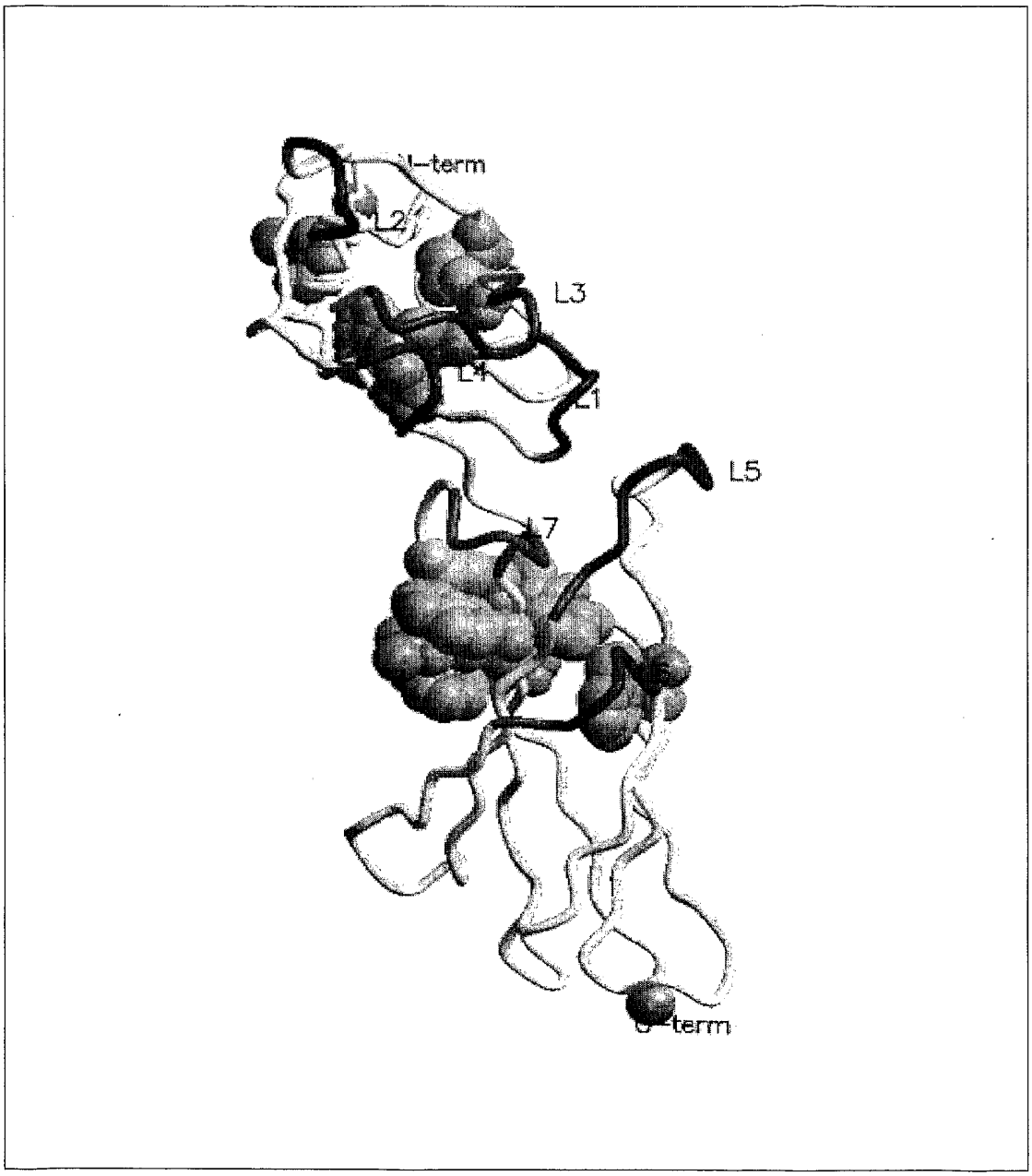


Figure 6(b)

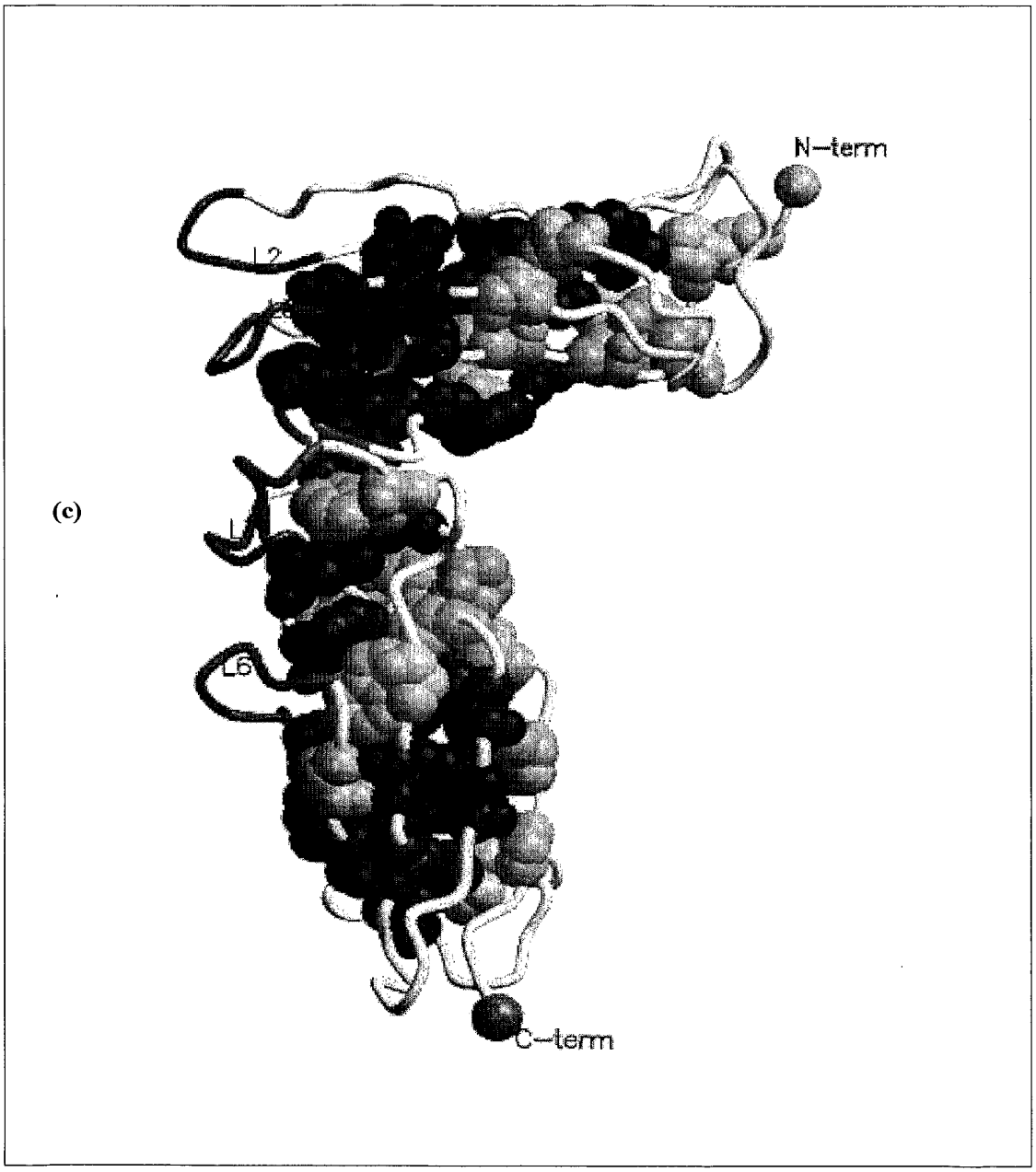


Figure 6(c)

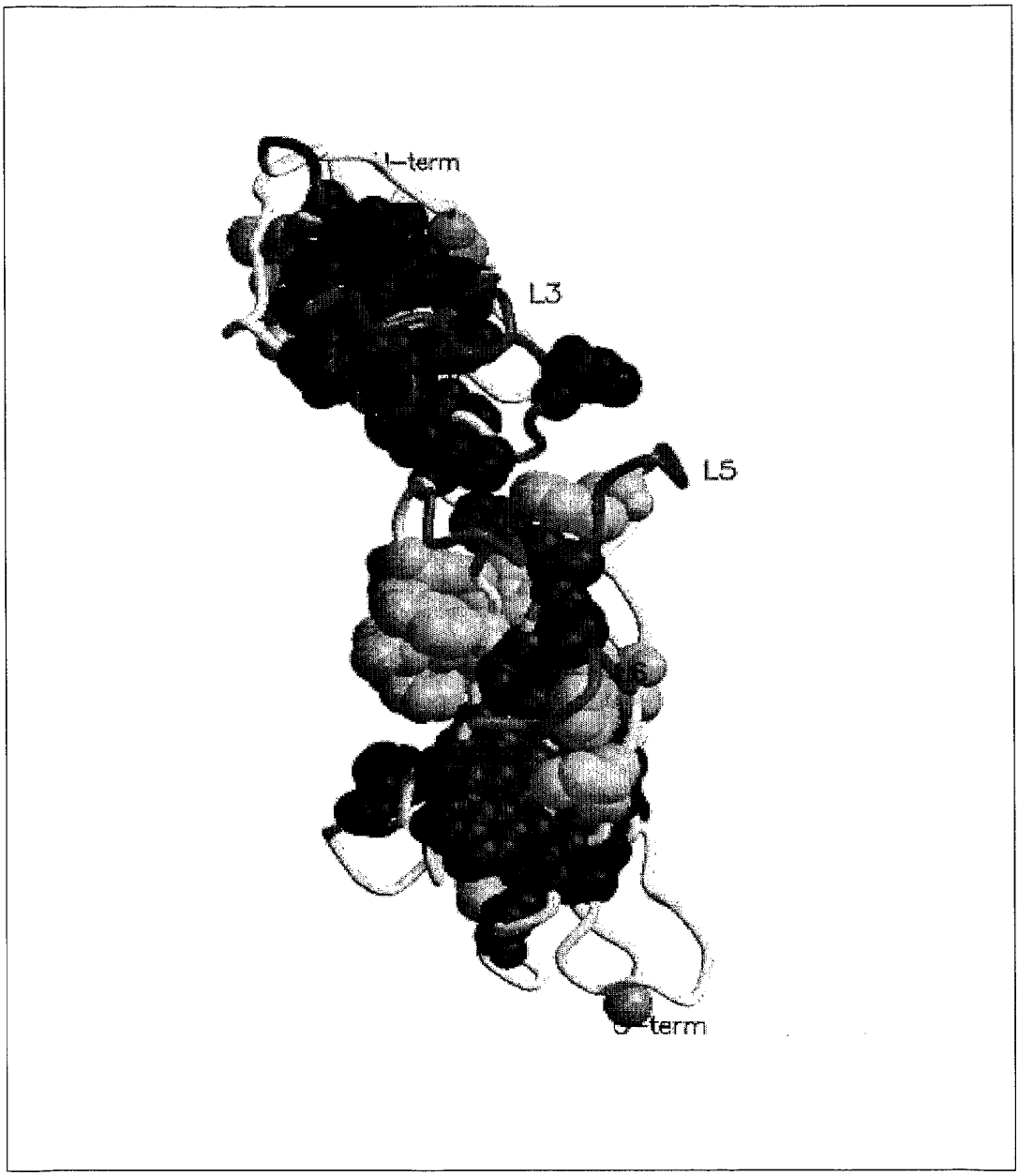


Figure 6(d)

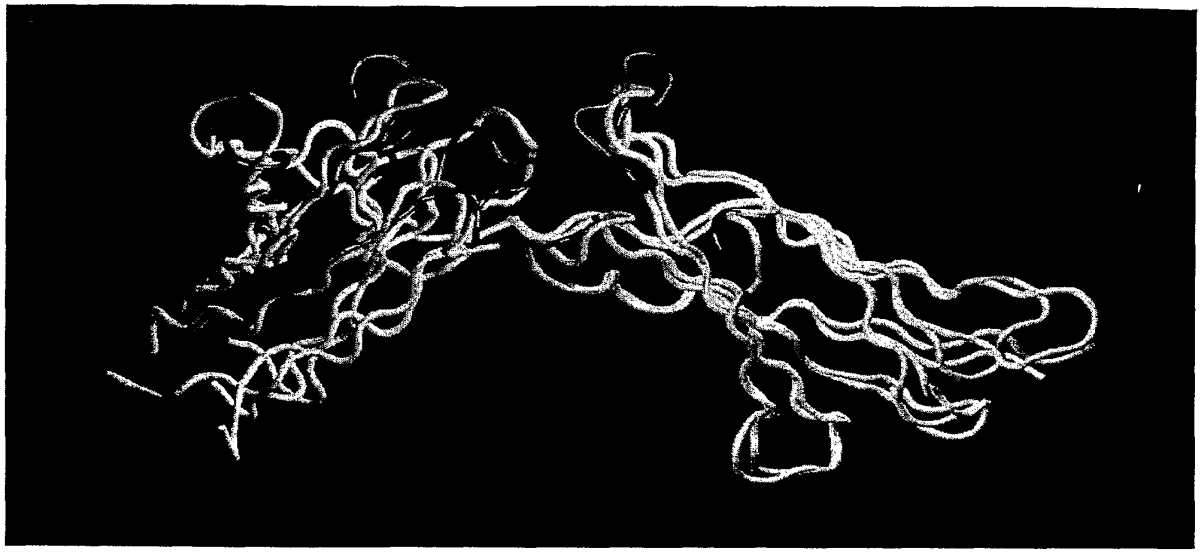


Figure 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/001762

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C07K 14/715, C12N 15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPIDS, MEDLINE, CAPLUS (cytokine, binding, domain, receptor, fibronectin, interleukin, modif, mutat, variant)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2002/032925 A2 (PHYLOS, INC.) 25 April 2002 (Whole document)	1, 2, 4-10, 16, 19, 20, 24-26, 29-33, 35-41, 50-52, 55-57, 60-67, and 69-88
X	Chuntharapai. A., et al: "Determination of residues involved in ligand binding and signal transmission in human IFN-α Receptor 2." The Journal of Immunology (1999); Vol 163: 766-773 (Whole document)	1, 2, 4-6, 11, 12, 19, 20, 31-33, 35-37, 42, 43, 50, 51 and 64-66.

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 January 2005	4 FEB 2005
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **Philippa Wyrdeman** Telephone No : (02) 6283 2554

Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/001762

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Gustin. S. E., et al; "Expression, crystallization and derivation of the complete extracellular domain of the β_c subunit of human IL-5, IL-3 and GM-CSF receptors." European Journal of Biochemistry (2001); Vol 268: 2905-2911 (Whole document)	1, 2, 4, 31-33, 35, 62 and 64-66.
X	Chill. J. H., et al; "The human type I interferon receptor: NMR structure reveals the molecular basis of ligand binding." Structure (July 2003), Vol 11: 791-802	1, 2, 5, 6, 11, 12, 19, 20, 31-33, 36, 37, 42, 43, 50 and 51

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/001762

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: **69-88**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
An essential technical feature of the invention appears to be the two FnIII-like domains with mutations in them. The claims, however, are not limited to this feature of the invention, they simply define any cytokine domain irrespective of whether there are two FnIII-like domains.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2004/001762

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	0232925	AU	13251/02	CA	2418835	EP	1356075

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX

line stripe and longitudinal columns are formed. By contrast, in midbrain development, *SHH* expression fans out from the ventral midline, and arcuate territories in register with the morphogen source are the result.

References and Notes

1. L. Wolpert, *J. Theor. Biol.* **25**, 1 (1969).
2. L. Wolpert, *Trends Genet.* **12**, 359 (1996).
3. J. Briscoe, J. Ericson, *Semin. Cell. Dev. Biol.* **10**, 353 (1999).
4. H. Roelink *et al.*, *Cell* **76**, 761 (1994).
5. E. Marti, D. A. Bumcrot, R. Takada, A. P. McMahon, *Nature* **375**, 322 (1995).
6. T. Sanders, A. Lumsden, C. Ragsdale, in preparation.
7. The *SHH* expression plasmid contained a cDNA encoding the 19.2-kD NH_2-terminal fragment of chick SHH [R. D. Riddle, R. L. Johnson, E. Laufer, C. Tabin, *Cell* **75**, 1401 (1993)] inserted into the expression vector pXeX [A. D. Johnson, P. A. Krieg, *Dev. Genet.* **17**, 280 (1995)]. Our results are based on whole-mount in situ hybridization analysis of 794 embryos transgenic for *SHH* expression. In 101 of the brains, *SHH* or its transcriptional target *FOXA2* (*8*) was detected directly to assess the size and location of the *SHH* source. No conclusion was based on a sample size of less than eight embryos. A human placental alkaline phosphatase expression vector [S. Fields-Berry, A. Halliday, C. L. Cepko, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 693 (1992)] was electroporated into 202 control embryos. Embryos damaged by electroporation were excluded from these sums. We injected 50 to 250 nl of plasmid DNA into the midbrain vesicle of stage 8 to 15 chick embryos [V. Hamburger, H. L. Hamilton, *J. Morphol.* **88**, 49 (1951)] and transfected the midbrain by conventional macroelectroporation [T. Muramatsu *et al.*, *Biochem. Biophys. Res. Commun.* **233**, 45 (1997); K. Sakamoto *et al.*, *FEBS Lett.* **426**, 337 (1998)] or by microelectroporation [T. Momose *et al.*, *Dev. Growth Differ.* **41**, 335 (1999)].
8. Y. Echelard *et al.*, *Cell* **75**, 1417 (1993).
9. V. Marigo, M. P. Scott, R. L. Johnson, L. V. Goodrich, C. J. Tabin, *Development* **122**, 1225 (1996).
10. Y. Watanabe, H. Nakamura, *Development* **127**, 1131 (2000).
11. T. Lecuit, S. M. Cohen, *Development* **125**, 4901 (1998).
12. J. B. Gurdon, P. Harger, A. Mitchell, P. Lemaire, *Nature* **371**, 487 (1994).
13. D. N. Keys *et al.*, *Science* **283**, 532 (1999).
14. H. F. Nijhout, *Dev. Biol.* **80**, 267 (1980).
15. K. Basler, G. Struhl, *Nature* **368**, 208 (1994).
16. B. A. Edgar, C. F. Lehner, *Science* **274**, 1646 (1996).
17. S. J. Day, P. A. Lawrence, *Development* **127**, 2977 (2000).
18. M. Placzek, J. Dodd, T. M. Jessell, *Curr. Opin. Neurobiol.* **10**, 15 (2000).
19. N. M. Le Douarin, M. E. Halpern, *Curr. Opin. Neurobiol.* **10**, 23 (2000).
20. E. A. Grove, S. Tole, J. Limon, L. Yip, C. W. Ragsdale, *Development* **125**, 2315 (1998).
21. Embryos were collected 30 min after an intravenous bromodeoxyuridine (BrdU) pulse and processed for in situ hybridization and antibody detection of BrdU-labeled cells.

22. We thank A. M. Greenlee for technical assistance, E. Ferguson, E. Grove, P. Mason, and V. Prince for discussions and C. Cepko, D. Engel, C. Goridis, M. Goulding, T. Jessell, A. Johnson, G. Martin, A. McMahon, and C. Tabin for cDNAs. Supported by National Institute of Neurological Disorders and Stroke (NIH).

26 December 2000; accepted 7 February 2001
Published online 1 March 2001;
10.1126/science.1058624
Include this information when citing this paper.

Structure of an Extracellular gp130 Cytokine Receptor Signaling Complex

Dar-chone Chow,[1] Xiao-lin He,[1] Andrew L. Snow,[1] Stefan Rose-John,[2] K. Christopher Garcia[1]*

The activation of gp130, a shared signal-transducing receptor for a family of cytokines, is initiated by recognition of ligand followed by oligomerization into a higher order signaling complex. Kaposi's sarcoma–associated herpesvirus encodes a functional homolog of human interleukin-6 (IL-6) that activates human gp130. In the 2.4 angstrom crystal structure of the extracellular signaling assembly between viral IL-6 and human gp130, two complexes are cross-linked into a tetramer through direct interactions between the immunoglobulin domain of gp130 and site III of viral IL-6, which is necessary for receptor activation. Unlike human IL-6 (which uses many hydrophilic residues), the viral cytokine largely uses hydrophobic amino acids to contact gp130, which enhances the complementarity of the viral IL-6–gp130 binding interfaces. The cross-reactivity of gp130 is apparently due to a chemical plasticity evident in the amphipathic gp130 cytokine-binding sites.

In vertebrates, gp130 is a shared signal-transducing receptor for a family of cytokines, including IL-6, herpesvirus IL-6 (vIL-6), IL-11, ciliary neurotrophic factor (CNTF), cardiotrophin (CT-1), leukemia inhibitory factor (LIF), oncostatin (OSM), and NNT-1/BSF3, which mediate a wide variety of both overlapping and unique biological responses in vivo (*1–4*). The activities of gp130 cytokines are mediated through formation of oligomeric complexes containing one or more copies of gp130, which leads to intracellular activation of Src and Janus tyrosine kinases and of the STAT family of transcription factors (*3, 5, 6*).

Cytokines that activate gp130 share a common, four-helix bundle fold (*7, 8*). Engagement of gp130 occurs through three conserved receptor-binding epitopes on the cytokines, the third of which is unique to gp130 cytokines (*9–13*). By analogy to other hematopoietic cytokine receptors, gp130 is presumed to recognize ligand through its cytokine-binding homology region (CHR), located at domains 2 and 3 (D2D3) (*8, 11–14*). However, gp130 uniquely requires an additional NH_2-terminal (D1) immunoglobulin (Ig)-like activation domain (IGD) in order to be functionally responsive to cytokine (*15*).

Hematopoietic receptors such as human growth hormone (hGH) exhibit a simple activation model characterized by the homodimerization of two receptors by one cytokine molecule via binding sites I and II (*16, 17*). In contrast, for gp130, "recognition" and "activation" complexes are disparate hetero-oligomeric species that are formed in a stepwise fashion (*11–13, 18, 19*). IL-6, the most extensively studied of the gp130 cytokines, cannot bind gp130 unless it first forms a complex with a specific α receptor (termed IL-6Rα, hereafter simply Rα) through a "site I" epitope (*3*). This binary IL-6–Rα complex forms a composite epitope, termed "site II," which interacts with the CHR of gp130 (D2D3 domains) to form a trimolecular (1:1:1) recognition complex, which is not competent for signaling. A transition to a higher order signaling assembly requires recruitment of the site III epitope and IGD into the recognition complex to form the higher order activation complex (*10, 15, 20, 21*). Although the topology of the activated assembly remains unknown, functional studies indicate that IL-6 and IL-11 signaling complexes are "hexamers" containing two copies each of cytokine, Rα, and gp130 (2:2:2) (*18, 22, 23*). Because the functional epitopes (sites I, II, and III) of all gp130 cytokines are in similar locations, it is likely that each signaling assembly will be constructed from a common oligomeric template.

Kaposi's sarcoma–associated herpesvirus (KSHV, or HHV8) is a recently discovered γ-herpesvirus that is a likely causative factor for the development of acquired immunodeficiency syndrome–related Kaposi's sarcoma (KS), as well as other neoplastic diseases associated with KS (*24*). KSHV encodes a functional homolog of interleukin-6 (termed vIL-6, 25% sequence homology) that is expressed in KS-infected cells and is able to induce angiogenesis and hematopoiesis in IL-6–dependent cell lines (*25, 26*). vIL-6 direct-

[1]Department of Microbiology and Immunology and Department of Structural Biology, Stanford University School of Medicine, Fairchild D319, 299 Campus Drive, Stanford, CA 94305, USA. [2]Department of Biochemistry, Christian-Albrechts-Universität zu Kiel, Olshausenstraße 40, D-24098 Kiel, Germany.

*To whom correspondence should be addressed. E-mail: kcgarcia@stanford.edu

ly activates gp130 on cells without the requirement for an α-receptor (*26*). Nonetheless, vIL-6 activation of gp130 initiates similar acute-phase responses and downstream signaling cascades (*27*). Therefore, vIL-6 is potentially a paracrine growth factor in bone marrow, suggesting a molecular mimicry in the oncogenesis of some lymphoproliferative disorders.

Here, we describe the crystal structure of KSHV IL-6 in complex with an extracellular fragment (D1D2D3) of human gp130 at 2.4 Å. This complex clarifies the structural roles of the activation epitope (site III) on the cytokine and the activation domain (IGD) on gp130, as well as the architecture of the higher order, extracellular signaling assembly. The tetrameric vIL-6–gp130 complex provides a structural template to model the hexameric extracellular signaling assemblies for other members of the gp130 cytokine family. This structure also elucidates a basis for molecular mimicry of a human cytokine by a viral homolog, and reveals the unique characteristics of gp130 that enable its extensive cross-reactivity with a diverse set of cytokines.

We coexpressed a soluble tetrameric complex (2:2) of vIL-6 and the three NH_2-terminal domains (D1D2D3) of human gp130, representing the CHR (D2D3) plus IGD (D1), in insect cells (molecular mass 126 kD) (*28*). Using identical methods, we coexpressed a hexameric human IL-6 (huIL-6)–Rα–gp130 complex, which exhibited a 2:2:2 stoichiometry (mass 193 kD), indicating that the difference between the viral and human assemblies is the presence of the two Rα in the human complex (*28*). Hence, the Rα independence of vIL-6, previously demonstrated in cellular experiments (*26*), is recapitulated with our soluble molecules. Crystallization of a nonglycosylated form of the viral complex resulted in crystals from which a complete x-ray data set to 2.4 Å was collected and the structure was determined (Fig. 1 and Table 1) (*29*). The crystals are space group C_2, and the complex twofold axis is coincident with a crystallographic axis (Fig. 2).

The complex assumes a tetrameric arrangement of two vIL-6 molecules and two human gp130 receptors (Fig. 2) (approximate dimensions 95 Å by 56 Å by 65 Å). Each vIL-6 molecule bridges two different gp130 molecules, and each gp130 binds two vIL-6 molecules through structurally distinct binding epitopes. The complex is tethered through the interaction of one face of vIL-6 (site II) with gp130 (D2D3) (Figs. 2 and 3) and through a second vIL-6 epitope (site III) interacting with the D1 domain of a different gp130 (Figs. 2 and 4). The tetramer is held together entirely through ligand-specific interactions, and the D1 domain is essential for the formation of the activated, higher order

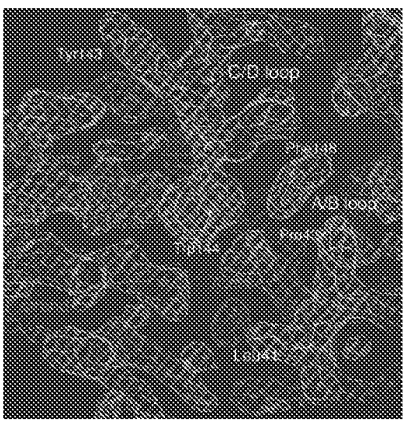


Fig. 1. Electron density in the site III interface of the vIL-6–gp130 complex. Viral IL-6 is in yellow and the gp130 D1 domain is in red. At the center of the map is vIL-6 Trp^{144}, which is at the core of the site III interface. The β-sheet strands of the gp130 D1 (F and G strands) are labeled in red. The electron density map is a SIGMAA-weighted $2F_{obs} - F_{calc}$ map at 2.4 Å contoured at 1.2σ and displayed with the program O (*45*).

Table 1. Crystallographic statistics for the complex between vIL-6 and gp130. KSHV vIL-6 in complex with the D1D2D3 domains of human gp130 was prepared by coinfection of sf9 cells with recombinant baculovirus secretion constructs of the individual proteins (*28*). Nonglycosylated vIL-6 and gp130 were produced by carrying out the expression in the presence of tunicamycin, an inhibitor of N-linked glycosylation (*29*). The secreted complexes were harvested from the supernatant and purified by Ni-NTA, gel filtration, and anion exchange chromatography (*28*). The nonglycosylated complex (10 mg/ml) crystallized from 10% MPEG-2K and 0.1 M Na citrate (pH 6.5) in space group C_2, with unit cell dimensions of a = 103 Å, b = 123.31 Å, c = 76.79 Å, β = 112.03°. A Matthews coefficient calculation (4.2 $Å^3$/dalton) indicated a 70% solvent content for one vIL-6 and one gp130 in the asymmetric unit (*45*). Therefore, a half-tetramer (or half-dimer) is present in the asymmetric unit, related by C_2 symmetry to the other half of the tetramer. The crystals were flash-frozen in liquid nitrogen in the presence of mother liquor containing 20% ethylene glycol, and data sets were collected at ALS beamline 5.02 and SSRL 9-2. A single data set from a crystal that diffracted to beyond 2.4 Å was collected, and integrated and scaled using MOSFLM and SCALA, respectively (*45*). Initial phases for two domains of gp130 were obtained by molecular replacement with AmoRe (*45*) using the coordinates of the CHR (D2D3) domain of gp130 (PDB ID: 1BQU) (*34*). For vIL-6, only MOLREP (*45*) succeeded in finding a molecular replacement solution using the coordinates of huIL-6 (PDB ID: 1ALU) (*37*) in which the residues were truncated to alanine. No model of an Ig domain gave a satisfactory solution for the gp130 D1 domain, so the structure of the D1 domain was traced from the electron density calculated with the partial structure of the refined D2D3 and vIL-6. The entire structure was then built using XFIT, as implemented in XTALVIEW (*45*). The structure was refined against a maximum likelihood target function, and consisted of a rigid-body refinement, several cycles of simulated annealing with torsion angle molecular dynamics, and iterations between positional and *B*-factor minimization. The structure was manually rebuilt using SIGMAA-weighted $2F_{obs} - F_{calc}$ and omit $F_{obs} - F_{calc}$ maps as implemented in CNS and the graphics program O (*45*), and stereochemical analysis was performed with PROCHECK (*45*). All data between 50 Å and 2.4 Å were used, except for 5% data randomly selected for cross-validation. All residues of the receptor (2 to 302) and the body of the cytokine (6 to 172) are ordered in the electron density (residues 2 to 6 and 173 to 180 are not visible in the vIL-6 electron density). The A/B loop of vIL-6, which is often disordered in uncomplexed cytokine crystal structures, is entirely ordered and exhibits average temperature factors. No evidence of N-linked glycosylation is present on either the cytokine or the receptor.

Data collection	
Resolution (Å) (highest resolution shell)	50.0 to 2.4 (2.53 to 2.40)
Measured reflections	106,397
Unique reflections	34,376
Completeness (%)	99.0 (99.6)
R_{merge} (%)*	8.1 (48.7)
$I/\sigma(I)$	3.4 (1.4)
Mosaicity (°)	0.4
Model refinement	
Resolution range (Å)	50.0 to 2.4 (2.53 to 2.40)
R_{cryst} (%)‡	21.3 (31.3)
R_{free} (%)§	25.6 (34.6)
No. of protein atoms	3783
No. of waters	370
Average *B* factor ($Å^2$)	
Protein main chain	46.3
Protein side chain	49.8
Water	54.9
RMSD angles (°)†	0.007
RMSD bonds (Å)†	1.6
Ramachandran plot (%)	
Most favored	87.9
Allowed	12.1

*$R_{merge} = \Sigma_{hkl}|I - \langle I\rangle|/\Sigma_{hkl}I$, where I is the intensity of unique reflection hkl, and $\langle I\rangle$ is the average over symmetry-related observation of unique reflection hkl. †The RMSD in bond lengths and angles is the RMSD from the ideal stereochemical values. ‡$R_{cryst} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. §R_{free} is R using a set of reflections sequestered before refinement.

assembly. The COOH-termini of each gp130 are oriented in similar directions, albeit with slightly divergent angles, where the three membrane-proximal domains not present in this structure would lead to the cell surface, as predicted from functional studies (*30, 31*). As seen from the top, the overall configuration results in a large hole in the middle of the complex (Fig. 2B) (~16 Å by 45 Å), a spacing likely critical for the gp130 extracellular domains to orient the intracellular domains correctly for signal propagation. The unused site I face of vIL-6 (B and D helices) is sterically accessible for engagement of an Rα, as would presumably occur in the human IL-6 hexamer.

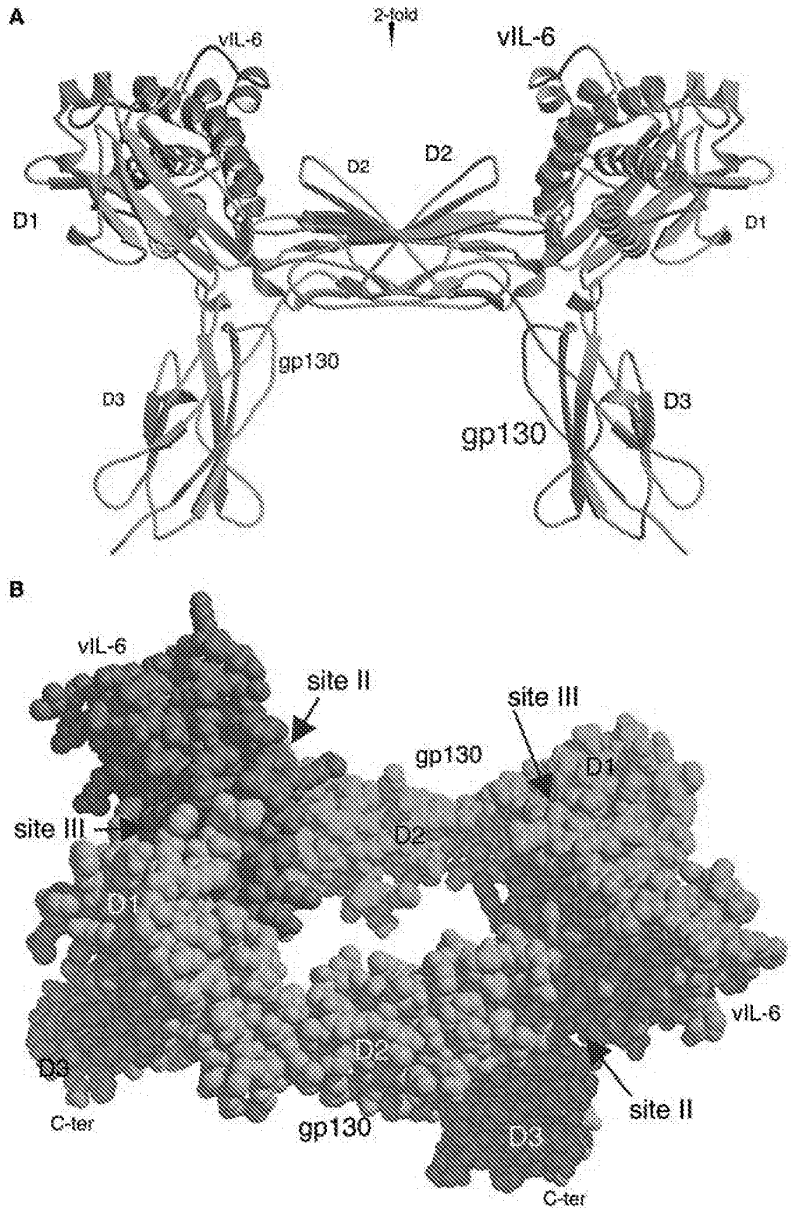


Fig. 2. Structure of the vIL-6–gp130 tetrameric signaling complex. Ribbon and space-filling models of vIL-6 complexed to gp130 (D1D2D3) in the 2:2 tetrameric oligomer are shown (gp130 molecules are blue and green, vIL-6 molecules are pink and red, and smaller and larger domain labels indicate farther and closer domains, respectively). (**A**) Side view of the complex; (**B**) a tilted view of a space-filling model. In (A), the COOH-termini of the gp130 D3 domains are at the bottom of the complex, pointing in the direction of the cell membrane. In this orientation, the second gp130 complex (in back) is obscured by the frontmost complex. When the side view is rotated toward the viewer by ~45°, the top of the complex is seen, and the second (back) receptor and cytokine from the side view (A) are now visible as related by the twofold axis, which runs through the hole in the center of the complex. The tilted view of the complex (B) enables the reader to simultaneously see the hole in the middle and the D3 domains underneath the "canopy" of vIL-6 and D1/D2 domains that dominate the uppermost portion of the complex. [Figures produced with Molscript and Raster3d (*45*).]

The extracellular fragment of gp130, comprising the NH_2-terminal D1, D2, and D3 domains, is extended (~115 Å long) but twisted at the domain boundaries (Fig. 2). D1 is a seven-stranded β-sandwich module that adopts a noncanonical h-type Ig fold (*32, 33*). The gp130 D1 contains an unusual disulfide bond at its NH_2-terminus between Cys^6 and Cys^{32}, which tethers the A strand to the B strand. The D1 domain is linked at an approximate 45° tilt angle to the D2 domain through a proline-rich linker, which has been compared to the linker between the V and C domains of an antibody. However, unlike the flexible "elbow angle" of antibodies, the gp130 D1 forms an extensive hydrophobic interface with D2, which restricts segmental flexibility between the domains and renders the D1 domain into a fixed position.

The D2D3, or CHR, module of gp130 is composed of two β-sandwich fibronectin type III domains connected at a tilt angle of ~80° to form an elbow. The structure is similar to a previously determined crystal structure of an unliganded gp130 D2D3 (*34*): There are essentially no conformational differences in the exposed loops and side chains of the cytokine-binding site [root mean square deviation (RMSD) = 0.72 Å for all Cα, 0.976 Å for all atoms]. Overall, gp130 appears to be a rigid structure that does not structurally adapt to cytokine, in contrast to other hematopoietic receptors that use conformational plasticity as a means of facilitating cross-reactivity (*35*).

The structure of vIL-6 shares the canonical up-up-down-down, ABCD four-helix bundle scaffold common to the "long chain" superfamily (Fig. 2A) (*8, 36*). The entire polypeptide chain is ordered in the electron density, largely as a result of packing of the A/B and C/D loops into the D1 domain of gp130 (Fig. 1) (these loops are often disordered in crystal structures of uncomplexed four-helix cytokines). Although only 25% homologous in sequence, vIL-6 most closely superimposes with huIL-6 (*37*), mainly because of the identical positions of two disulfide bonds (Cys^{31}-Cys^{37}, Cys^{60}-Cys^{70}) and the residues comprising the helical hydrophobic core (RMSD = 1.25 Å for 82 Cα, 1.7 Å total). Most of the amino acids in the hydrophobic core are conserved between human and viral IL-6. Hence, the viral cytokine has reproduced, with high fidelity, the helical scaffold on which to display alternative gp130 contact residues.

Although the structure of the huIL-6–gp130

complex is not known, it is clear that both site II and site III vIL-6 epitopes use different amino acids to contact gp130 than would be used by huIL-6. However, structure-based sequence alignment between vIL-6 and huIL-6 shows that contact residues seen in the structure of the vIL-6–gp130 complex are in the same positions as huIL-6–gp130 contact residues previously mapped by mutagenesis (*12*). Hence, the docking orientations of the human and viral cytokines on gp130 are likely identical at both sites II and III. This allows us to make direct comparisons of amino acid positions in each cytokine with the assumption that the human gp130 cytokine and viral complex templates are identical.

The site II contact surface between vIL-6 and the gp130 CHR is primarily composed of hydrophobic interactions between the A and C helices of vIL-6 and the CD and EF loops of the D2 domain and the BC loop of the D3 domain of gp130 (Fig. 3). Thirteen vIL-6 residues and 17 gp130 contact residues bury a total of 1194 Å^2, with only two hydrogen bonds in the interface (*38*). The two hydrogen bonds (Arg^{15}Nη2-Phe^{169}O, Asp^{100}Oδ1-Ser^{165}Oγ) may constrain the orientation of the gp130–vIL-6 crossing angle. There is excellent shape complementarity between the cytokine and receptor (Fig. 3, B and C), in which the protruding gp130 "elbow" slots into a diagonal groove on a concave face of vIL-6 created by the crossing angle of the A and C helices (Fig. 3B). A stripe of three tryptophans (Trp^{111}, Trp^{18}, and Trp^{21}) on vIL-6 diagonal to the cytokine helical axes (Fig. 3, B and C) defines the top, middle, and bottom of the cytokine groove and interacts, in order, with the top (CD), middle (EF), and bottom (BC) loops linking the gp130 CHR β-strand elements (Fig. 3).

The core of the interface is dominated by a hydrophobic cluster composed of Trp^{18} on the vIL-6 A helix (green patch in Fig. 3B, green amino acid in Fig. 3C) and Tyr^{168}-Phe^{169} (blue patch in Fig. 4B) on the EF loop of gp130 D2, and Val^{230} on the BC loop of gp130 D3 (*38*). The vIL-6 Trp^{18} and gp130 Phe^{169} protrude into mutually complementary pockets (Fig. 3, B and C). Mutation of gp130 Phe^{169} and Val^{230} is disruptive to interaction with gp130 cytokines, and large aromatics at gp130 positions 168 and 169 are highly conserved across species (*31, 34, 39*). Hence, the insertion of the receptor Phe^{169} into a pocket on the cytokine A/C face likely represents a common feature underlying gp130 cytokine recognition. These amino acids, then, may represent the energetic "hotspots" of the site II interface, as has been observed for a structurally analogous Trp residue (Trp^{104}) in the core of the hGH receptor interface (*40*).

None of the 13 vIL-6 site II residues in contact with gp130 are shared with huIL-6. The stripe of three tryptophans on vIL-6 (Trp^{18}, Trp^{21}, and Trp^{111}) is replaced by

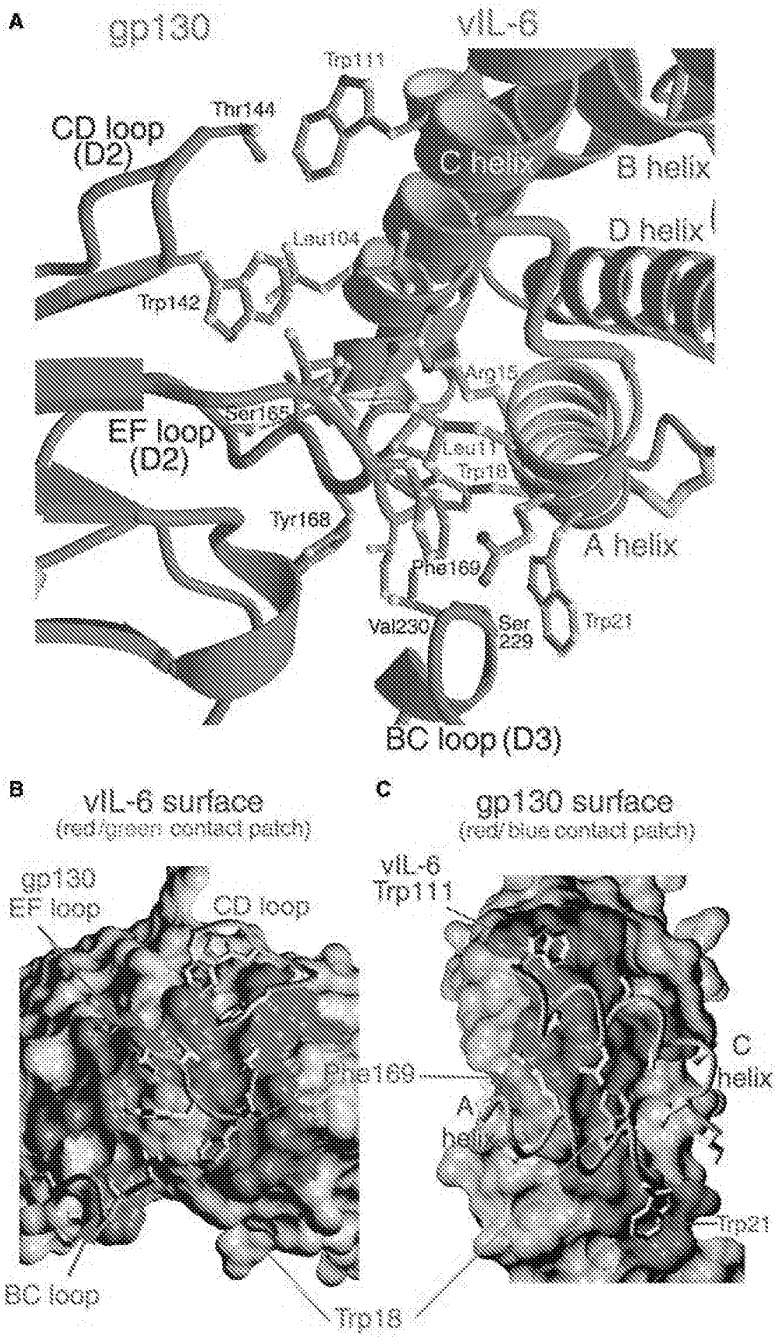


Fig. 3. Molecular anatomy and shape complementarity of the site II interface. (**A**) Amino acid contact residues within the site II interface; gp130 (blue) is at the left and vIL-6 (purple) at the right, and hydrogen bonds appear as red dotted lines. The conserved gp130 Phe^{169} and vIL-6 Trp^{18} are in the center of the interface. (**B** and **C**) The site II vIL-6–gp130 interface is peeled open, and the contact residues of one molecule are projected onto the buried surface (red) of the interacting protein. Trp^{18} of vIL-6 (green) and Phe^{169} of gp130 (blue) are used as anchor points (arrows) to orient the reader between the two surfaces. In (B), the gp130 contact residues (yellow) and binding-site loops (blue) are shown as sticks projected onto the molecular surface of vIL-6, where the buried portion is highlighted in red. A deep canyon that receives the protruding gp130 elbow is evident on the surface of vIL-6. The central Trp^{18} of vIL-6 is highlighted on the red surface as a green patch. In (C), the vIL-6 contact residues (yellow) and helices (pink) are drawn as sticks projected onto the protruding molecular surface of gp130. The Phe^{169} of gp130 is drawn as a blue patch [see (*38*)]. [Figures produced with Molscript, Raster3d, and VMD (surface calculated with MSMS using 1.4 Å probe radius) (*45*).]

Tyr[31], Asp[34], and Gln[124], respectively. Hence, the three largest hydrophobic contact residues in vIL-6 are replaced by smaller, more polar amino acids in huIL-6, which would reduce the shape complementarity of this interface and rationalizes, in part, the huIL-6 requirement for its α-receptor (Rα) to stabilize binding to a nonoptimal gp130 D2D3 binding site. The increased size, hydrophobicity, and shape complementarity of key contact positions in vIL-6 site II renders an Rα dispensable.

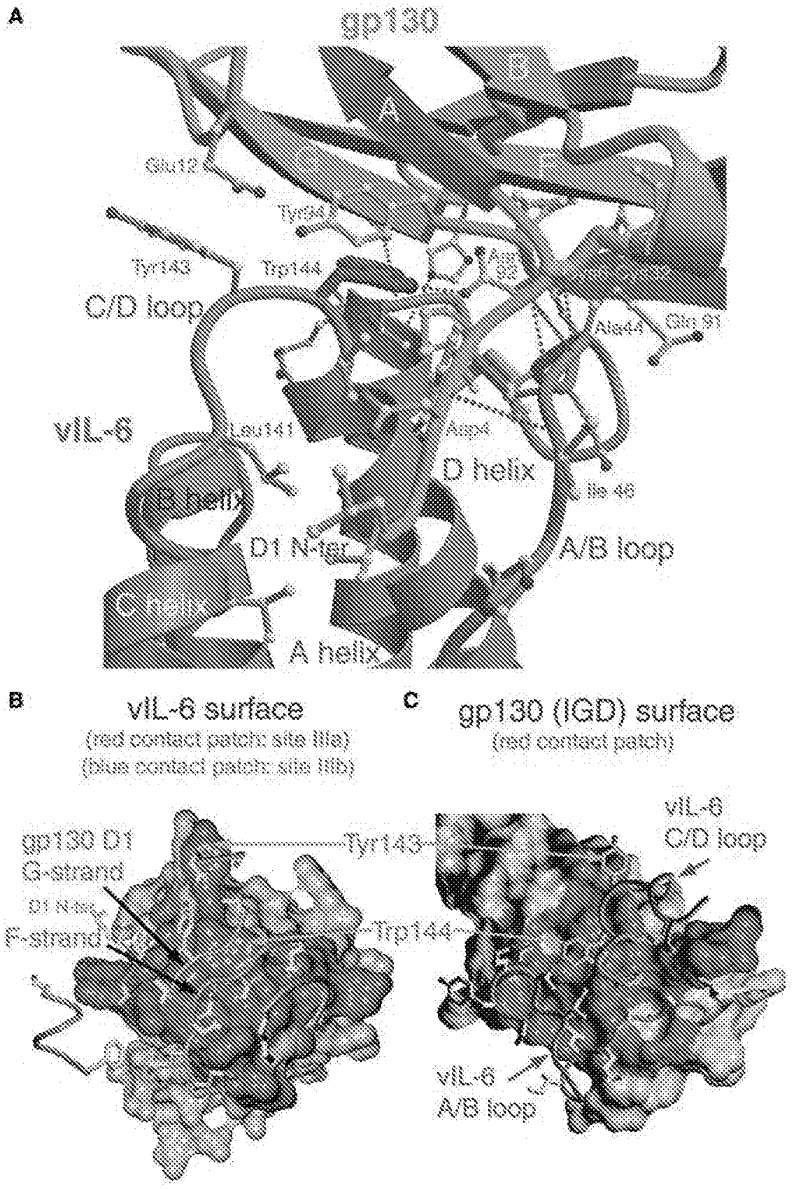


Fig. 4. Molecular anatomy and shape complementarity of the site III interface. (**A**) Amino acid contact residues within the site III interface; vIL-6 (magenta) is at the bottom and the gp130 D1 (IGD) domain (green) at the top, and hydrogen bonds appear as red dotted lines. The hallmark site III residue of vIL-6, Trp[144], is drawn in purple with a thicker stick rendering. β-sheet strands of the gp130 IGD are labeled as indicated. (**B** and **C**) The site III vIL-6–gp130 interface is peeled open, and the contact residues of one molecule are projected onto the buried surface (red) of the interacting protein. Tyr[143] and Trp[144] of vIL-6 are used as anchor points (blue arrows) to orient the reader between the two surfaces. In (B), the vIL-6 contact residues (yellow for site IIIa, blue for site IIIb) and binding-site loops are shown as sticks projected onto the molecular surface of the gp130 IGD where the buried portion is highlighted in red. In (C), the gp130 IGD contact residues (yellow) and β-sheet strands (green) are drawn as sticks projected onto the protruding site III molecular surface of vIL-6. The total site III contact surface of vIL-6 is divided into site IIIa (red) and site IIIb (blue) to indicate the relative locations of side chains within the surface. The strand designations of the IGD β-sheet strands are labeled.

The molecular basis of the striking cross-reactivity of gp130 for a diverse set (15 to 20% sequence homology) of cytokines likely resides in a combination of highly solvent-exposed hydrophobic residues in the gp130 CHR binding site (Fig. 3, A and B) and an amphipathic binding surface capable of both nonpolar and polar interactions. The solvent accessibility of nonpolar residues on the surface of the gp130 site II implies a favorable entropic driving force for shielding this surface from solvent through burial by cytokine, and the lack of side chain–specific polar interactions may lead to promiscuity, as has been suggested for the binding site of human Ig-Fc (*41*). The cross-reactivity of gp130 is further enhanced by the amphipathic nature of the site II and III surfaces, which enables the accommodation of a spectrum of binding chemistries.

All gp130 cytokines possess a third (site III) functional epitope, which is necessary for receptor activation, but the nature of its interaction with gp130 has been unclear (*10, 13*). In the structure of the vIL-6–gp130 complex, the site III interaction comprises an extensive interface between the tips of the vIL-6 four-helix bundle (A/B loop and start of D helix) and the edge (G and F strands) of the upper three-stranded β sheet of the gp130 IGD (D1) domain (Figs. 1 and 4) (*38*). The complementary shape of the interface is formed by the convex tip of vIL-6 resting in a depression formed by the curvature of the upper D1 β sheet (Fig. 4, A and C). The interface is discontinuous and is composed of two separate structural elements on vIL-6: residues in the A/B loop (site IIIa) and the start of the D helix (site IIIb) (Fig. 4) (*38*). These loops primarily interact with the G β strand, and to a lesser extent with the F, A, and c′ strands of the receptor D1 Ig β sandwich (Fig. 1). This region of an Ig domain has not been previously seen as a ligand-binding site. A total of 1641 Å^2 of surface is buried in the interface and is partitioned on vIL-6 into 60% from the A/B loop (site IIIa) and 40% from the D helix (site IIIb) (*38*). The core of the interface is formed by vIL-6 site IIIb hydrophobic residues Tyr[143], Trp[144], and Phe[148] packing into a depression on the surface of the G strand. In site IIIa, the vIL-6 A/B loop, usually disordered in uncomplexed cytokines, forms an almost continuous interaction with D1 through numerous main-chain H-bonds (Fig. 4A). The NH_2-terminal four residues of D1, which precede the unusual disulfide (Cys[6]-Cys[32]) between strands A and B, form a strand-like interaction with A/B loop residues 44 to 49 of vIL-6 (Fig. 4A). Although the functional importance of this contact is unknown, modifications to the NH_2-terminus of gp130 disrupt signaling (*42*).

Extensive mutagenesis data on gp130 cyto-

kines implicate a large hydrophobic residue at the NH_2-terminus of the D helix as being critical to a functional site III (*9, 10, 18, 43*). In particular, a critical Trp^{157} in huIL-6 (Phe^{160} in OSM, CNTF, and LIF) is present at the identical position (Trp^{144}) in the vIL-6 structure, placing it in the center of the interface (Figs. 1 and 4), where it buries the largest fraction of surface area of all residues in site III and forms a hydrogen bond with Asn^{92} of D1 (*38*). Hence, the vIL-6 molecular mimicry of the huIL-6 site III is achieved through preservation of an identical central core residue (Trp^{144}) that likely is the "hotspot" of the interface, but a different set of peripheral contact residues is presented (9 of 13 vIL-6 contact residues differ).

The site III binding region on the gp130 D1 contains both degenerate and specific structural features relevant to its function as a shared signal transducer. D1 is a rigid structural framework that does not appear to use conformational plasticity to cross-react with multiple different cytokine site III surfaces. Rather, the site III interaction surface of the cytokine is composed primarily of flexible interhelical loops, and it appears to adapt its structure to the surface of D1 in an induced-fit type of interaction. The predominance of main-chain H-bonds between the A/B loops (site IIIa) and D1 also enhances the promiscuity of the site III–D1 interface (Fig. 4). Similar to site II, the D1 binding site uses amphipathicity to broaden the range of ligand surface chemistries with which it can interact, with residues such as Tyr^{94} and Asn^{92} participating in both polar and nonpolar interactions (the gp130 binding surface is 58% polar, 42% nonpolar; the vIL-6 buried surface is 16% polar, 84% nonpolar).

Our biochemical studies using a soluble D1D2D3 fragment of gp130 indicate that the human IL-6 signaling assembly consists of a "hexamer" containing two huIL-6, two Rα, and two gp130 (2:2:2), which is consistent with data from functional studies using full-length receptor (*18, 22, 23, 44*). In the structure of the vIL-6–gp130 tetramer, the outward helical face of vIL-6 (B and D helices), where huIL-6 would interact with Rα (site I), is unoccupied but is openly accessible in the complex (Fig. 2), clarifying the location of Rα in the human signaling complexes. For the huIL-6 hexamer, the two site I faces will likely each be occupied by an Rα in a manner similar to the site I interaction between hGH and GHR, creating the hexameric structure. This hexamer model directly applies to human IL-11 and IL-12 signaling complexes, as they both dimerize shared signal transducers. The resolution of how gp130 cytokines such as LIF, CNTF, OSM, and CT-1 heterodimerize their receptors, breaking the hexameric symmetry, remains a challenge for future structural studies of this important cytokine superfamily.

References and Notes

1. M. Hibi *et al.*, *Cell* **63**, 1149 (1990).
2. T. Kishimoto, T. Taga, S. Akira, *Cell* **76**, 253 (1994).
3. T. Taga, T. Kishimoto, *Annu. Rev. Immunol.* **15**, 797 (1997).
4. G. Senaldi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 11458 (1999).
5. J. N. Ihle, B. A. Witthuhn, F. W. Quelle, K. Yamamoto, O. Silvennoinen, *Annu. Rev. Immunol.* **13**, 369 (1995).
6. P. C. Heinrich, I. Behrmann, G. Muller-Newen, F. Schaper, L. Graeve, *Biochem. J.* **334**, 297 (1998).
7. J. F. Bazan, *Immunol. Today* **11**, 350 (1990).
8. S. R. Sprang, J. F. Bazan, *Curr. Opin. Struct. Biol.* **3**, 815 (1993).
9. J. Brakenhoff *et al.*, *J. Biol. Chem.* **269**, 86 (1994).
10. M. Inoue *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 8579 (1995).
11. J. Grotzinger, G. Kurapkat, A. Wollmer, M. Kalai, S. Rose-John, *Proteins* **27**, 96 (1997).
12. R. J. Simpson, A. Hammacher, D. K. Smith, J. M. Matthews, L. D. Ward, *Protein Sci.* **6**, 929 (1997).
13. J. Bravo, J. K. Heath, *EMBO J.* **19**, 2399 (2000).
14. J. F. Bazan, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6934 (1990).
15. A. Hammacher *et al.*, *J. Biol. Chem.* **273**, 22701 (1998).
16. J. A. Wells, A. M. de Vos, *Annu. Rev. Biochem.* **65**, 609 (1996).
17. I. A. Wilson, L. K. Jolliffe, *Curr. Opin. Struct. Biol.* **9**, 696 (1999).
18. G. Paonessa *et al.*, *EMBO J.* **14**, 1942 (1995).
19. D. Staunton, K. R. Hudson, J. K. Heath, *Protein Eng.* **11**, 1093 (1998).
20. I. Kurth *et al.*, *J. Immunol.* **162**, 1480 (1999).
21. K. J. Kallen *et al.*, *J. Biol. Chem.* **274**, 11859 (1999).
22. L. D. Ward, G. J. Howlett, A. Hammacher, R. L. Moritz, R. J. Simpson, *Ann. N.Y. Acad. Sci.* **762**, 471 (1995).
23. V. A. Barton, M. A. Hall, K. R. Hudson, J. K. Heath, *J. Biol. Chem.* **275**, 36197 (2000).
24. E. Cesarman, Y. Chang, P. S. Moore, J. W. Said, D. M. Knowles, *N. Engl. J. Med.* **332**, 1186 (1995).
25. P. S. Moore, C. Boshoff, R. A. Weiss, Y. Chang, *Science* **274**, 1739 (1996).
26. S. H. Hoischen *et al.*, *Eur. J. Biochem.* **267**, 3604 (2000).
27. J. Molden, Y. Chang, Y. You, P. S. Moore, M. A. Goldsmith, *J. Biol. Chem.* **272**, 19625 (1997).
28. The proteins and complexes for these studies were produced using the baculovirus system in insect cells (Baculogold-Pharmingen). We amplified and subcloned the D1D2D3 domains of gp130 (amino acid residues 2 to 303) and the mature coding sequence of KSHV IL-6 (residues 2 to 180) into the pAcgp67A secretion vector (Pharmingen); 6-histidine tags were appended to the COOH-termini. Sf9 cells were transfected and recombinant virus was amplified using standard methods. For large-scale expression of the complexes, Tn5 cells grown to a density of 2×10^6 cells per milliliter in Insect-Xpress (BioWhittaker) in a shaking Fernbach flask were simultaneously infected with recombinant virus from both gp130 and vIL-6 at a multiplicity of infection of at least 10. Coinfection substantially increased the yield of complex versus expression of each individual component and subsequent mixing. Cultures were allowed to progress for 48 to 60 hours before the cells were pelleted by centrifugation, the supernatant was clarified by filtration, and the volume of the supernatant was reduced by tangential flow concentration. The complexes were captured by Ni-NTA resin and then purified by gel filtration chromatography (Superdex-200) and/or mono-Q fast protein liquid chromatography (Pharmacia). The vIL-6 and gp130 formed a stoichiometric, stable complex whose mass was determined by gel filtration and analytical ultracentrifugation to be ~126 kD (theoretical mass, 131.5 kD). This mass indicates the presence of a 2:2 ratio of gp130 to vIL-6. Human IL-6–Rα and gp130 formed an equally stable complex of ~193 kD, indicating a 2:2:2 stoichiometry (theoretical mass, 191.6 kD) (D. C. Chow *et al.*, unpublished data). The mass difference between the vIL-6–gp130 and huIL-6–Rα–gp130 complexes (~60 kD) is accounted for by the 2 Rα ($2 \times$ ~30 kD) not present in the vIL-6 complex.
29. The complexes expressed from insect cells are heavily glycosylated. The gp130 D1D2D3 domains contain six potential N-linked glycosylation sites, and the vIL-6 contains two. Our enzymatic deglycosylation experiments indicate that all sites are used for carbohydrate addition. We crystallized the fully glycosylated complex; however, these crystals, although ordered, failed to diffract x-rays beyond 8 to 9 Å at synchrotron x-ray sources. Therefore, we expressed the complex in the presence of a known N-linked glycosylation inhibitor, tunicamycin. Addition of tunicamycin (0.2 μg/ml) to the expression media immediately before infection resulted in the production of complexes free of N-linked carbohydrate. This material was purified by methods identical to those used for the glycosylated material, and in all respects it behaved identically to the glycosylated complex. We grew crystals of this material that diffracted x-rays to beyond 2.0 Å resolution, and from which a complete 2.4 Å data set was collected.
30. A. Hammacher *et al.*, *Biochem. J.* **345**, 25 (2000).
31. I. Kurth *et al.*, *J. Immunol.* **164**, 273 (2000).
32. The D1 domain of gp130 can be classified as an h-type Ig domain according to the classification criteria of Bork (*33*). There is a 4-on-3 sheet arrangement of the order a-b-e-d on c-c′-f-g. Although there is a short c′ strand, which would place it in the v-type fold, there are only 12 amino acids between the start of the c strand and the start of the e strand, placing it in the h-type category. A DALI search of the D1 resulted in the highest Z-scores for both v- and h-type Ig folds [L. Holm, C. Sander, *J. Mol. Biol.* **233**, 123 (1993)]. Hence, the gp130 D1 is best considered a hybrid of a v- and h-type Ig fold, with a short c-c′ strand.
33. P. Bork, L. Holm, C. Sander, *J. Mol. Biol.* **242**, 309 (1994).
34. J. Bravo, D. Staunton, J. K. Heath, E. Y. Jones, *EMBO J.* **17**, 1665 (1998).
35. S. Atwell, M. Ultsch, A. M. De Vos, J. A. Wells, *Science* **278**, 1125 (1997).
36. J. F. Bazan, *Neuron* **7**, 197 (1991).
37. W. Somers, M. Stahl, J. S. Seehra, *EMBO J.* **16**, 989 (1997).
38. See *Science* Online (www.sciencemag.org/cgi/content/full/291/5511/2150/DC1).
39. U. Horsten *et al.*, *J. Biol. Chem.* **272**, 23748 (1997).
40. T. Clackson, J. A. Wells, *Science* **267**, 383 (1995).
41. W. L. DeLano, M. H. Ultsch, A. M. de Vos, J. A. Wells, *Science* **287**, 1279 (2000).
42. R. L. Moritz *et al.*, *Growth Factors* **16**, 265 (1999).
43. M. C. Deller *et al.*, *Struct. Fold Des.* **8**, 863 (2000).
44. D. C. Chow *et al.*, unpublished data.
45. Crystallographic and graphics programs used were ALSCRIPT [G. J. Barton, *Protein Eng.* **6**, 37 (1993)]; CCP4, AmoRe, MOSFLM, SCALA [CCP4, *Acta Crystallogr.* **D50**, 760 (1994)]; MOLREP [V. Vagin, A. Teplyakov, *J. Appl. Crystallogr.* **30**, 1022 (1997)]; XTALVIEW (XFIT) [D. E. McRee, *J. Struct. Biol.* **125**, 156 (1999)]; CNS [A. T. Brünger *et al.*, *Acta Crystallogr.* **D54**, 905 (1998)]; MOLSCRIPT [P. J. Kraulis, *J. Appl. Crystallogr.* **24**, 946 (1991)]; RASTER3D [E. A. Merrit, M. E. P. Murphy, *Acta Crystallogr.* **D50**, 869 (1994)]; VMD [W. Humphrey, A. Dalke, K. Schulten, *J. Mol. Graphics* **14**, 33 (1996)]; MSMS [M. F. Sanner, J.-C. Spehner, A. J. Olson, *Biopolymers* **38**, 305 (1996)]; Matthews Coefficient [B. W. Matthews, *J. Mol. Biol.* **33**, 491 (1968)]; PROCHECK [R. A. Laskowski, M. W. MacAuthur, D. S. Moss, J. M. Thornton, *J. Appl. Crystallogr.* **26**, 283 (1993)]; and O [T. A. Jones, J. Y. Zhou, S. W. Cowan, M. Kjeldgaard, *Acta Crystallogr.* **A47**, 110 (1991)].

46. We thank F. Bazan, W. Weis, and R. Kastelein for helpful discussions; J. Ho, J. Coller, and B. Orman for technical assistance; the staff of the Advanced Light Source (G. McDermott and T. Earnest); and the staff of the Stanford Synchrotron Radiation Laboratory (SSRL) (M. Soltis and P. Kuhn). Supported by a postdoctoral training grant (NIH) to the Department of Microbiology and Immunology (D.-c.C.), the California Cancer Research Program and American Heart Association (X.-L.H.), the Stanford Immunology Program (A.L.S.), and the Cancer Research Institute, the California Cancer Research Program, a Rita Allen Foundation award, NIH (grant RO1-AI-48540-01), and the Stanford University School of Medicine (K.C.G.). Coordinates are available from the Protein Data Bank (PDB) under accession code 1I1R. Coordinates of the tetramer are also available from the authors (kcgarcia@stanford.edu).

14 December 2000; accepted 6 February 2001

Structure of an Extracellular gp130 Cytokine Receptor Signaling Complex

Dar-chone Chow, Xiao-lin He, Andrew L. Snow, Stefan Rose-John and K. Christopher Garcia

Science **291** (5511), 2150-2155.
DOI: 10.1126/science.1058308

Cytokine & Growth Factor Reviews 26 (2015) 489–498

Contents lists available at ScienceDirect

Cytokine & Growth Factor Reviews

journal homepage: www.elsevier.com/locate/cytogfr

Mini review

Emerging roles for IL-11 signaling in cancer development and progression: Focus on breast cancer

Cameron N. Johnstone [a,b,c], Ashwini Chand [a,b], Tracy L. Putoczki [d,e], Matthias Ernst [a,b,*]

[a] Cancer & Inflammation Laboratory, Olivia Newton-John Cancer Research Institute, Level 5, Olivia Newton-John Cancer & Wellness Centre, Heidelberg, VIC 3084, Australia
[b] School of Cancer Medicine, LaTrobe University, Heidelberg, VIC 3084, Australia
[c] Cancer Metastasis Laboratory, Cancer Research Division, Peter MacCallum Cancer Centre, East Melbourne, VIC 3002, Australia
[d] Inflammation Division, Walter & Eliza Hall Institute of Medical Research, Parkville, VIC 3052, Australia
[e] Department of Medical Biology, Melbourne University, Parkville, VIC 3052, Australia

ARTICLE INFO

Article history:
Received 8 July 2015
Accepted 8 July 2015
Available online 14 July 2015

Keywords:
Cancer
Interleukin
IL-6
IL-11
STAT3

ABSTRACT

Interleukin (IL)-11 is a member of the IL-6 family of cytokines that is defined by the shared use of the GP130 signal transducing receptor subunit. In addition of its long recognized activities as a hemopoietic growth factor, IL-11 has an emerging role in epithelial cancer biology. Through the activation of the GP130-Janus kinase signaling cascade and associated transcription factor STAT3, IL-11 can confer many of the tumor intrinsic 'hallmark' capabilities to neoplastic cells, if they express the ligand-specific IL-11Rα receptor subunit. Accordingly, IL-11 signaling has recently been identified as a rate-limiting step for the growth tumors arising from the mucosa of the gastrointestinal tract. However, there is less appreciation for a potential role of IL-11 to support breast cancer progression, apart from its well documented capacity to facilitate bone metastasis. Here we review evidence that IL-11 expression in breast cancer correlates with poor disease outcome and discuss some of the molecular mechanisms that are likely to underpin these observations. These include the capacity of IL-11 to stimulate survival and proliferation of cancer cells alongside angiogenesis of the primary tumor and of metastatic progenies at distant organs. We review current strategies to interfere with IL-11 signaling and advocate that inhibition of IL-11 signaling may represent an emerging therapeutic opportunity for numerous cancers.

1. Introduction

Interleukin (IL)-11 is a member of the IL-6 family of cytokines that comprises nine secreted soluble ligands; IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin-M (OSM), ciliaryneurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), interleukin-27 (IL-27) and interleukin-31 (IL-31) [1–10]. Each ligand interacts with a specific non-catalytic transmembrane receptor or receptors, commonly referred to as the receptor alpha chain. Importantly, the family is defined by their shared use of the ubiquitously expressed transmembrane protein glycoprotein-130 beta-subunit (GP130, also known as IL6ST or CD130) [4,11,12].

IL-11 was purified as 19 kDa soluble factor in supernatants from a stromal cell line that promoted the proliferation of a plasmacytoma cell line that was otherwise dependent on IL-6 [10]. Although crystallization of the 178 amino acid human IL-11 protein revealed the characteristic type 1 four-helix bundle, its structure shows some distinct differences to that of its closest relative IL-6 [13]. Meanwhile the 7 kb human *IL11* gene comprises five coding exons and is localized to chromosome 19q13.3–19q13.4 [14].

Traditionally, IL-11 is recognized for its capacity to promote maturation of platelet producing megakaryocyte progenitors *in vitro* and in the bone marrow *in vivo* [15,16]. In order to boost platelet production, numerous clinical trials have been conducted with recombinant IL-11 in patients with breast cancer to reduce thrombocytopenia associated with chemotherapy [17,18]. This culminated in the recent FDA approval of Oprelvekin, as a modified more stable form of IL-11, to reduce chemotherapy-induced thrombocytopenia at lower doses [19,20]. Despite these striking activities within the hematopoietic system, studies in knockout

* Corresponding author at: Cancer & Inflammation Laboratory, Olivia Newton-John Cancer Research Institute, Level 5, Olivia Newton-John Cancer & Wellness Centre, 145 Studley Road, Heidelberg, VIC 3084, Australia.
E-mail address: matthias.ernst@onjcri.org.au (M. Ernst).

http://dx.doi.org/10.1016/j.cytogfr.2015.07.015

mice have revealed that IL-11 is not essential for hematopoiesis, but instead is critical within the endometrial tissue of the pregnant adult female, as genetic deficiency of the IL-11 receptor subunit prevented formation of a decidua and hence resulted in abortion of 5 day old mouse embryos [21]. More recently, the use of *Il11ra*-null mice has revealed an unprecedented role for IL-11 signaling as a "gate keeper" for the growth of adenomas and possibly more advanced tumors derived from the gastrointestinal mucosa [22], and these findings have recently been reviewed [1]. Here we will focus on emerging insights into the role of IL-11 in the development and progression of breast cancer.

2. IL-11 signaling

IL-11 binds to its specific transmembrane receptor, IL-11 receptor alpha (IL-11Rα) [23]. It is believed that the IL-11/IL-11Rα dimeric complex interacts in turn with GP130 potentially as a tetrameric complex [24] (Fig. 1). In turn, and in analogy to IL-6 signaling, it is believed that these complexes transition into a high affinity ligand-binding and signaling-proficient hexameric complex comprising a 2:2:2 ratio of ligand, IL-11Rα and GP130 [25]. The formation of this larger order complex initiates signaling through juxta-positioning of the intracellular Janus (JAK) family tyrosine kinases JAK1, JAK2 and TYK2, which are constitutively associated with a proline-rich intracellular domain of GP130, and enables kinase activation in response to *trans*-phosporylation [12]. Activated JAK kinases in turn phosphorylate the various cytoplasmic tyrosine residues in GP130 to provide docking sites for the signaling molecules signal-transducer and activated of transcription-3 (STAT3), STAT1, SHP2 and the negative regulator suppressor of cytokine signaling (SOCS3), in addition to JAK-mediated tyrosine phosphorylation of the former molecules [12]. Genetic evidence suggests that activation of STAT3 is the most important event for the transduction of a majority of biological responses to GP130-family cytokines [26] (Fig. 2). While most of these effects depend on transcriptional regulation of target genes upon the binding of tyrosine-phosphorylated STAT3-dimers to regulatory DNA sequences, non-canonical activities of serine-phosphorylated STAT3 also appear to promote its oncogenic capacity by facilitating glycolysis and oxidative phosphorylation in the mitochondria [27]. However, most of tumor-cell intrinsic 'Cancer Hallmark' activities elicited by STAT3 depend on its canonical role as a transcriptional modulator of target genes that affect cell proliferation, survival, motility and invasion [28,29] (Fig. 2). A key STAT3-induced target gene encodes the SOCS3 protein, which terminates GP130 signaling. SOCS3 binds to a membrane-proximal phosphotyrosine residue in GP130 to mediate formation of an E3 ligase scaffold with elongin BC and a cullin protein resulting in ubiquitination of the receptor complex and its proteasomal degradation [30].

Besides activation of STAT3, and to a lesser extent also STAT1, engagement of GP130 also triggers signaling through the RAS–RAF–ERK pathway following GP130 association and subsequent JAK-dependent phosphorylation of the tyrosine phosphatase SHP2/PTPN11 [31]. Finally, GP130 has also the capacity to activate the phosphatidylinositol 3′ kinase (PI3K)–AKT–mTORC1 pathway, although in contrast to engagement of the STAT and SHP2/ERK signaling cascades, the former does not require tyrosine phosphorylation of GP130 [32] (Fig. 2).

3. Expression of IL-11 and relationship to outcome in breast cancer

3.1. IL-11 expression

Carcinomas comprise neoplastic epithelial cells intermingled with various non-transformed stromal cell types (Fig. 1). Individually, and in concert with one another, these non-transformed cell types collectively comprise the tumor microenvironment, and influence most if not all aspects of cancer cell behavior. In turn, tumor cells influence the composition and function of the tumor microenvironment [33,34]. Although expression of IL-11 has not been evaluated as extensively as that for IL-6 in whole breast tumors, most information regarding cell-type specific expression can be extrapolated from studies of other cancer types [35], or from analysis of human breast cancer cell lines [36–41]. In the majority of primary tumor lesions of all breast cancer sub-types and stages, IL-11 expression is elevated when compared to adjacent normal breast tissue, (Fig. 3A, B). Furthermore, analysis of expression

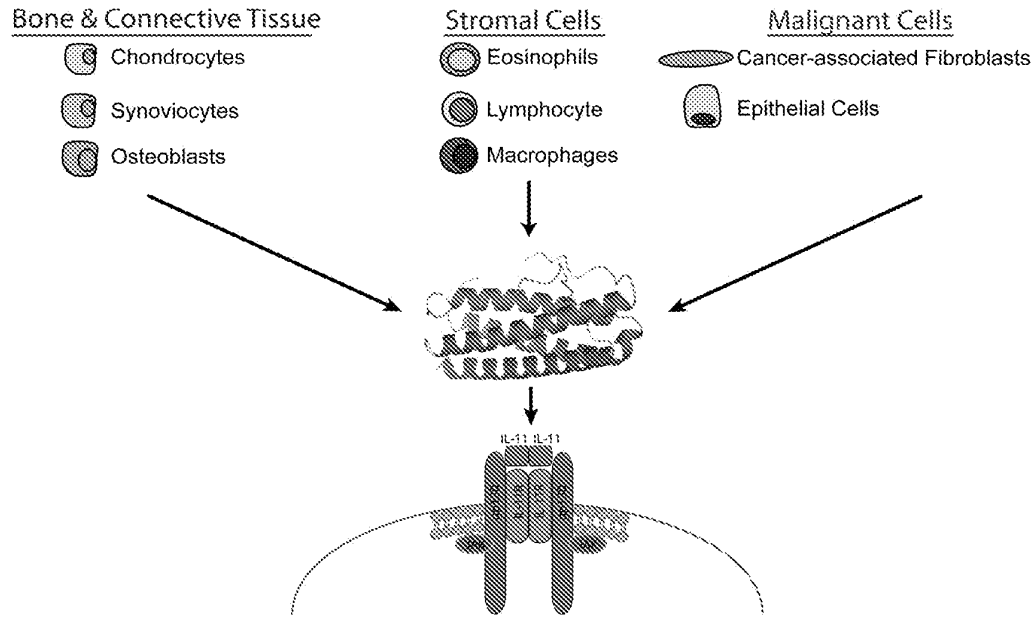


Fig. 1. Schematic representation of the major the cellular sources of IL-11, which forms a hexameric 2:2:2 signaling complex comprising IL-11, IL-11Rα and GP130.

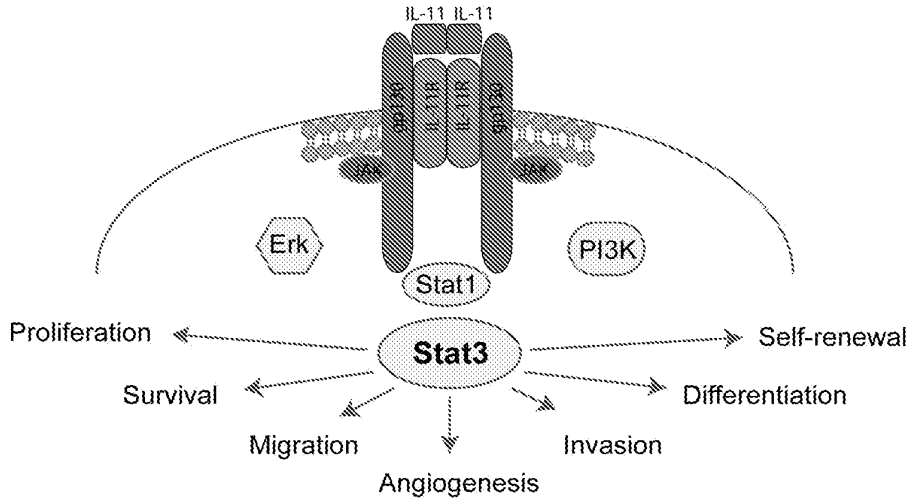


Fig. 2. Schematic representation of the pro-tumourigenic activities elicited through the IL-11/GP130/STAT3 signaling cascade.

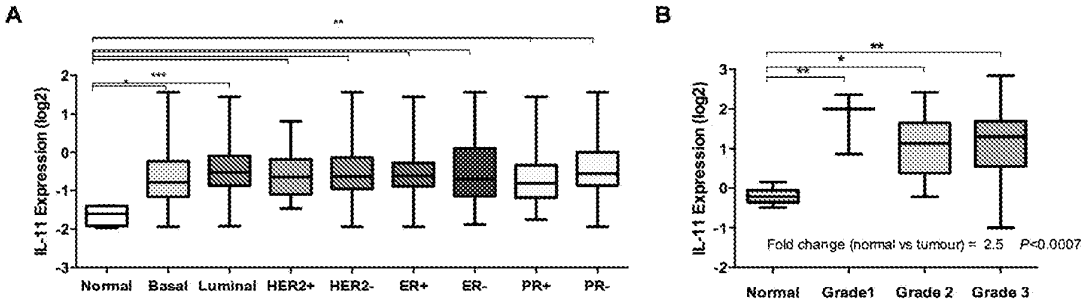


Fig. 3. IL-11 is elevated in breast cancer tissue irrespective of grade or hormone receptor status. (A) 'Gluck' [119] and (B) 'Finak' [120] Oncomine data is presented. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.001$.

profiling data from The Cancer Genome Atlas (TCGA) and from the Curtis datasets through the Oncomine portal (http://www.oncomine.org, [42]) revealed elevated IL-11 transcript levels in both primary ductal and lobular breast adenocarcinoma (Table 1), suggestive of a potential role in breast carcinogenesis [43,44].

In the healthy mouse, IL-11 mRNA is detected at low abundance in thymus, spleen, heart, gastrointestinal tract, kidney, brain, testis, uterus, ovaries and bones [45,45], where production increase in response to infection, injury and inflammation [46]. Subepithelial myofibroblasts are a major source of IL-11 [47], as well as the gastrointestinal epithelium and both cell types are likely to contribute IL-11 to cancers alongside the carcinoma cells, tumor-associated macrophages (TAMs) and T cells [2] (Fig. 1). In a neo-adjuvant trial of breast cancer patients undergoing chemotherapy, administration of epirubicin plus cyclophosphamide plus docetaxel enhanced IL-11 expression in primary breast tumors (Table 2), although no further investigations were carried out to determine the cellular origin of IL-11 [48]. However, this observation was not confirmed in a similar larger chemotherapy neo-adjuvant trial [49]. In response to chemotherapy, expression of IL-6, but not IL-11, was induced in thymic endothelial cells in a mouse model of Burkitt lymphoma and promoted survival of

Table 1
Expression of IL-11 and IL-11Rα mRNA in normal breast and breast cancer: an analysis using Oncomine data.

Gene	Type	F.C.	*P*	Sample size	Platform	Refs.
IL11	I.D.C.	1.75	5.80E−11	Normal (61), cancer (389)	RNA-Seq	TCGA [43]
IL11	I.L.C.	1.46	1.32E−4	Normal (61), cancer (36)	RNA-Seq	TCGA [43]
IL11	I.D.C.	1.04	1.43E−7	Normal (144), cancer (1556)	Array	Curtis [44]
IL11	I.L.C.	1.02	n/s	Normal (144), cancer (148)	Array	Curtis [44]
IL11	Mucinous	1.01	n/s	Normal (144), cancer (46)	Array	Curtis [115]
IL11RA	I.D.C.	−3.87	2.35E−51	Normal (61), cancer (389)	RNA-Seq	TCGA [43]
IL11RA	I.L.C.	−2.69	1.24E−17	Normal (61), cancer (36)	RNA-Seq	TCGA [116]
IL11RA	I.D.C.	−3.33	9.47E−117	Normal (144), cancer (1556)	Array	Curtis [44]
IL11RA	I.L.C.	−2.49	2.25E−54	Normal (144), cancer (148)	Array	Curtis [44]
IL11RA	Mucinous	−3.81	2.20E−21	Normal (144), cancer (46)	Array	Curtis [44]

P values were determined using Student's *t*-test.
F.C., fold change; Refs., references; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; TCGA, The Cancer Genome Atlas.

Table 2
Association of IL-11 mRNA levels in primary breast ductal tumors with patient outcome and other clinical parameters: an analysis using Oncomine data.

Gene	Parameter	F.C.	*P*	*N*	Platform	Refs.
IL11	Neo-chemo	2.61	7.43E−8	Control (32), treated (25)	Array	Stickeler [48]
IL11	3 yr RFS	1.77	4.23E−5	No relapse (124), relapse (32)	Array	Desmedt [63]
IL11	3 yr DMFS	1.77	2.00E−4	No metastasis (138), metastasis (19)	Array	Desmedt [63]
IL11	5 yr RFS	1.53	0.002	No relapse (105), relapse (47)	Array	Desmedt [63]
IL11	5 yr DMFS	1.44	0.022	No metastasis (123), metastasis (29)	Array	Desmedt [63]

P values were determined using Student's *t*-test.
F.C., fold change; *N*, sample size; Neo-chemo, Neo-adjuvant chemotherapy; RFS, relapse-free survival; DMFS, distant metastasis-free survival; yr, year; Refs., references.

residual tumor cells in the thymus [50]. IL-6 was also induced in doxorubicin-treated human umbilical vein endothelial cells, suggesting that the vascular endothelium may also be a potential source of IL-6 related cytokines in certain clinical contexts.

In colorectal tumors, carcinoma-associated fibroblasts (CAFs) appear to be the principal source of IL-11, where *IL11* gene expression was activated in response to exposure to tumor cell-derived TGF-β [35]. The latter is consistent with the identification of two AP-1 motifs in the 5′ region of the *IL11* gene that are essential for TGFβ1-induced transcriptional activation. Meanwhile, additional *cis*-regulatory elements in the gene promoter comprise, among others, binding sites for SP-1, STAT3, CTF/NF-1 and possibly NFκB thereby confirming a role for IL-11 during inflammatory processes and both autocrine and paracrine enforcement of STAT3-dependent signaling [10,51].

3.2. IL-11Rα expression

Interrogation of microarray data reveals relatively broad expression of IL-11Rα mRNA in breast cancer cell lines (Johnstone C.N., unpublished observation), which we also observe in human breast tissue biopsies (Fig. 4). Published investigations at the protein and transcript level showed minimal IL-11Rα expression in normal mammary gland, although expression of IL-11Rα alongside its ligand were increased in a subset of primary breast cancers [40,52]. However, expression of IL-11Rα was down-regulated in the ductal, lobular and mucinous histotypes of human breast cancer (Table 1). The former comprises the most common histological subtype and is further divided into the estrogen receptor alpha (ERα)-positive, HER2-positive and triple-negative (negative for ERα, progesterone receptor (PR) and HER2) clinical subtypes [44]. A recent meta-analysis of 21 breast cancer gene expression datasets proposed that triple-negative tumors can be further divided [53]. This includes a mesenchymal stem cell-like (MSL) subgroup, which is characterized by IL-11Rα expression as an identifying marker [53]. This subtype has undergone epithelial-to-mesenchymal transition (EMT) and is particularly aggressive with a poor patient outcome [53,54]. Since EMT often correlates with therapy resistance, it has been suggested that the breast cancer prognostic marker miRNA-30c targets IL-11 expression *via* an actin binding protein [55]. High miRNA-30c expression inversely correlated with low IL-11 expression and improved survival in breast cancer patients [55]. Surprisingly, despite accumulation of SOCS3 transcripts [36,37], triple negative tumors show very low levels of SOCS3 protein [56], possibly resulting in sustained JAK–STAT signaling. Thus, similar to observations in other epithelial cancers, IL-11Rα expression is down-regulated in most primary breast tumors with the notable exception of elevated expression in the MSL-like subgroup of triple-negative cancers.

3.3. IL-11 signaling and patient outcome

Levels of IL-11 transcripts, rather than of IL-11Rα transcripts, generally correlate positively with breast cancer progression. These associations are reminiscent of reports for hepatocellular carcinoma [57], gastric carcinoma [58] and renal cell carcinoma [59], and more recently IL-11 has also been implicated in poor survival of patients with non-small cell lung adenocarcinoma [60]. Similarly, there is also a trend toward higher IL-11 expression in tumors from patients with local or distant recurrence, as well as in patients who died from breast cancer, although these associations did not reach statistical significance possibly due to the small sample size [52]. Meanwhile, Sotiriou et al. [61] reported in a cohort of 89 patients a statistically highly significant association between IL-11 expression in primary tumors and an increased risk for development of bone metastasis. In support of this, IL-11 protein levels in the serum and primary tumors were significantly higher in patients with bone metastasis compared to those without distant metastasis [62]. Moreover, in the cases with bone metastasis, serum IL-11 levels were positively associated with reduced disease-free survival [62]. Similarly, higher IL-11 transcript levels have been observed in breast cancer patients that relapsed 3–5 years after initial diagnosis when compared to a relapse-free cohort [63]. While there is less compelling evidence between IL-11 expression and breast cancer patient outcome using

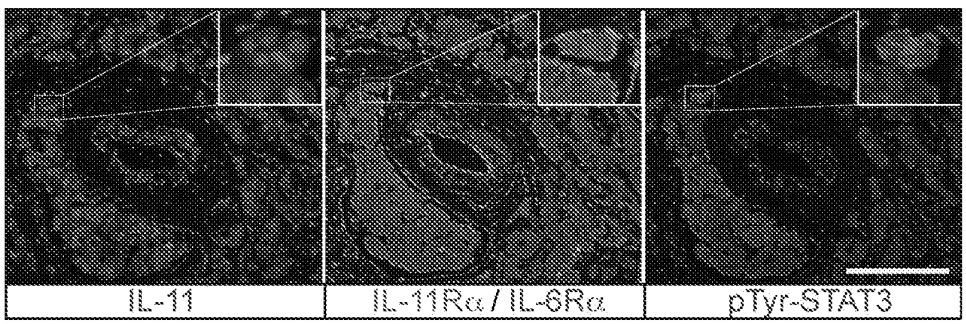


Fig. 4. IL-11 is produced by a range of cell populations in human primary breast tumors (left panel), with IL-6R and IL-11Rα expression (middle panel) primarily associated with mammary epithelial cells and STAT3 activation (right panel). Scale bar = 100 μm.

Table 3
Association of IL-11 and IL11RA mRNA levels in primary breast tumors with patient outcome and other clinical parameters: Kaplan–Meier analyses.

Gene	Subgroup	Dataset	Survival	H.R.	95% C.I.	*N*	*P*	Refs.
IL11RA	L.N. metastasis negative	26 datasets	DFS	0.7961	0.6433–0.9852	1183	0.0357	Madden [64]
IL11	All	Cardiff	DFS	NR	NR	?	0.0100	Hanavadi [52]
IL11	All	UNC337	RFS[a]	2.3763	NR	337	0.0002	Bockhorn [55], Prat [117]
IL11	All	Oxford	DMFS[b]	2.6620	NR	210	0.0210	Bockhorn [55], Buffa [118]
IL11	All	UC	PFS[b]	1.7524	NR	44	0.0375	Bockhorn [55]

H.R., hazard ratio; C.I., confidence interval; *N*, sample size; Refs., references; L.N., lymph node; DFS, disease-free survival; RFS, relapse-free survival; DMFS, distant metastasis-free survival; PFS, progression-free survival; NR, not reported.
[a] Gene expression values were dichotomized and allocated into high and low groups split at the median.
[b] Gene expression values were used as a continuous variable.

the BreastMark meta-analysis algorithm (http://glados.ucd.ie/BreastMark/index.html) to survey survival data from 26 datasets on 12 different microarray platforms [64], such data may be limited by the frequent incorrect annotation of IL-11/IL-11Rα on some microarray platforms. Nevertheless, BreastMark analysis revealed an association between higher IL-11Rα expression and prolonged patient survival specifically in lymph node metastasis-negative patients (Table 3).

4. Molecular activities of IL-11 in breast cancer

4.1. Proliferation and Apoptosis

IL-11 acts as a potent growth factor for various hematopoietic progenitors, and the growth of tumors of the gastrointestinal mucosa is fueled by IL-11 [22], consistent with the capacity of STAT3 to transcriptionally induce genes that promote cell cycle progression (Fig. 2). Likewise, a second cancer hallmark activity of STAT3, namely to promote cellular survival through both the induction of Bcl-2 survival proteins as well as, possibly through indirect mechanisms, suppression of their BH3-only protein antagonists, is a prominent IL-11 activity [22]. Indeed we have identified the latter as the major mechanism that confers resistance of the colonic epithelium to experimentally induced acute colitis, as well as a mechanism by which IL-11 antagonists suppress the growth of gastrointestinal tumors in mice [22]. Consistent with these observations, Bockhorn et al. [55] also found a role for IL-11 in chemotherapy-induced apoptosis of MDA-MB-231 triple-negative breast cancer cells. Specifically, siRNA-mediated knockdown of IL-11 sensitized the cells to paclitaxel-induced cell death, while anti-IL-11 antibody added to culture medium increased doxorubicin-induced apoptosis. Indeed higher IL-11 expression was associated with reduced progression-free and overall survival in a cohort of 25 patients treated with doxorubicin, suggesting that IL-11 may also antagonize pro-apoptotic pathways *in vivo* [55]. Whether this is limited to the cell intrinsic apoptotic pathway or may also affect the tumor necrosis factor (TNF) superfamily dependent extrinsic pathway, remains to be fully elucidated.

4.2. Tumor hypoxia and angiogenesis

Cancers require a vascular network to both provide nutrients for growth and to remove harmful by products of proliferation and cellular metabolism. Indeed, stimulation of new blood vessels (angiogenesis) is required if tumors are to reach a size greater than a few mm^3 [65]. Hypoxia near the center of actively growing tumors results in stabilization of the transcription factor hypoxia-inducible factor-1α (HIF-1α), which in concert with STAT3 activates expression of the key angiogenic growth factor vascular endothelial growth factor (VEGF) to stimulate angiogenesis [66]. Interestingly, IL-11 rather than IL-6 was induced by hypoxia in a range of cancer cell lines [67]. Induction by hypoxia was mediated by the HIF-1α and AP-1 transcription factors. Similarly, *in vitro* experiments showed that IL-11 expression enhanced the tumorigenicity of PC-3 prostate cancer cells under hypoxic, but not normoxic, conditions and this was associated with STAT1 rather than STAT3 phosphorylation [67]. Consistent with this, short-hairpin RNA (shRNA)-mediated stable knockdown of IL-11 attenuated the growth of PC-3 xenografts [67]. Furthermore, loss of IL-11 increased apoptosis of tumor cells primarily at early time points, consistent with a key role for IL-11 in the enhanced survival of tumors while emerging in a hypoxic microenvironment. Interestingly, while these investigators did not observe differences in microvessel density in IL-11 silenced tumors, others reported that exclusion of the IL-11-producing tumor subclone in a polyclonal xenograft was associated with a reduction of vessel growth [68]. Human umbilical vein endothelial cells express IL-11Rα and respond to stimulation with IL-11 [69], thus raising the possibility that IL-11 may have direct effects on tumor endothelium.

4.3. Cancer stem cells and tumor heterogeneity

An emerging concept in breast cancers and tumors of other origins is the dynamic equilibrium between cancer stem cells (CSCs) and their non-cancer stem cells (NCSCs) progenies [70]. CSCs have the ability to initiate *de novo* tumors when transplanted, whereas NCSCs do not [70]. Since CSCs often divide more slowly than the bulk population of the tumor, they may escape chemotherapy-induced cell death and enable tumor recurrence at a later time point. At the molecular level, breast cancer stem cells are defined as bearing a $CD44^+CD24^-$ surface marker phenotype [71], or being positive for the enzyme aldehyde dehydrogenase (ALDH1) [72], and both of these populations form tumors in mouse models. Phenotypically, breast CSCs have been described as undifferentiated, or possessing a mesenchymal phenotype [73], and thus might be generated from NCSCs by epithelial-to-mesenchymal transition [74]. Indeed, Iliopoulos et al. [75] have shown that the conversion of NCSCs to CSCs is enhanced by IL-6. Several other studies identified critical roles for the JAK-STAT pathway and canonical STAT3 signaling in the maintenance of the CSCs population in breast cancer [76]. The STAT3 inhibitors LLL12 or STATTIC, for instance, reduced the viability of breast CSCs and inhibited the growth of xenograft tumors in mice that were formed by an inoculum enriched in CSCs [77]. Complementary studies have shown that IL-6 signaling mediates stem cell maintenance through STAT3-dependent mechanisms [78,79]. Polyak and colleagues delineated an IL-6/JAK2/STAT3 pathway in breast CSCs where the JAK2-specific inhibitor NVP-BSK805 caused tumor regression in triple-negative tumor xenografts in mice [79]. Meanwhile in the HER2+ breast cancer subtype, resistance often emerges to the standard-of-care anti-HER2 antibody (Trastuzumab) adjuvant therapy through mutational activation of the PI3K/AKT signaling pathway [80]. Since the latter results in increased production of IL-6, IL-8 and CCL5 [81] it is likely to also induce expression of IL-11, although this was not experimentally confirmed. Elevated IL-6 secretion stimulated the expansion of

the CSC population in several human breast cancer models, through engagement of the downstream JAK/STAT3 pathway, and is also likely to be associated with NFκB signaling, which is an inducer of IL-6. Importantly, growth of Trastuzumab-resistant tumors was entirely dependent on sustained IL-6 signaling, as administration of the anti-IL-6R antibody Tocilizumab eliminated tumor growth *in vivo* [81].

Tumor heterogeneity and clonal cooperation are important concepts in our understanding that within a lesion the cancer cells may behave as a community and collectively confer increased aggressiveness to polyclonal tumors. Using a candidate approach of overexpressing cancer-promoting factors in individual subclones of MDA-MB-468 triple-negative breast cancer cells, Marusyk and colleagues identified IL-11 and vascular endothelial growth factor-D (VEGF-D) as the two factors that reproducibly conferred metastatic behavior to the corresponding polyclonal xenografts [68]. Furthermore, the IL-11 expressing subclone(s) acted as non-cell autonomous driver(s) of xenograft growth, most likely also promoting expansion of the VEGF-D producing clone to ensure sufficient (lymph-)angiogenesis. Indeed, the continuous presence of IL-11 appeared to ensure that all subclones maintained a certain equilibrium, which collectively retained aggressiveness of the xenograft. Removal of the IL-11 producing clone, on the other hand, prevented further growth of the xenografts and often resulted in their necrotic collapse. Surprisingly, neither IL-11 (nor VEGF-D) acted directly on the cancer cells. These data suggest a hitherto unrecognized capacity whereby IL-11 in the tumor microenvironment may not only promote tumor progression by maintaining an equilibrium among the most aggressive subclones, but also controls tumor angiogenesis, which may occur by both direct and indirect mechanisms [82].

4.4. Aromatase expression

Aromatase (*CYP19A1*) belongs to the cytochrome P450 group of enzymes and catalyzes the biosynthesis of estrogens. While in premenopausal women, the ovaries have the highest aromatase expression [83], in post-menopausal women with breast cancers, aberrant transcription of the *CYP19A1* gene in CAFs increases intratumoral estrogen levels and promotes tumor growth [84]. Accordingly, aromatase inhibitors are currently used to treat Tamoxifen-resistant ERα-positive tumors. While the mechanism for enhanced *CYP19A1* transcription in response to tumor-secreted factors involves the PII gene promoter normally utilized for ovarian transcription [85], the adipose tissue-specific PI.4 promoter contributes to local *CYP19A1* expression in the tumor microenvironment [86]. PI.4-mediated transcription is stimulated by TNFα and, in synergy with the activated glucocorticoid receptor, the GP130 cytokines IL-11 and oncostatin-M [87]. Consistent with this the PI.4 promoter contains specific GAS sequence to facilitate STAT1/3 binding, and its close proximity to a glucocorticoid response element molecularly underpins the synergistic effect between dexamethasone and IL-11 stimulated aromatase expression [87–89]. It remains to be established whether this mechanism also contributes to the breast cancer risk posed by chronic and obesity-associated inflammation, because accumulation of CD68+ macrophages in breast adipose tissue of obese women correlates with increased aromatase levels [90]. Thus, tumor-associated macrophages, *via* the secretion of cytokines such as oncostatin-M and IL-11, may contribute to elevated aromatase activity in breast cancer.

4.5. Metastatic dissemination

While IL-11 is likely to be only a lesser driver of cancer cell proliferation [35,39,91,92], evidence has accumulated for IL-11 to facilitate metastatic dissemination of cancer cells to distant sites. As mentioned above, clinical data strongly implicates IL-11 in metastasis to bone. IL-11 is produced by bone marrow stromal cells where it can stimulate osteoclast development from progenitor cells [93] (Fig. 1). Unlike in prostate cancer, where the bone lesions are osteoblastic, breast cancer bone metastases are usually osteolytic, resulting in net bone destruction through breast cancer cell-released mediators, including IL-11 and parathyroid hormone-related protein (PTHrP), which activate osteoclasts [94].

Osteoclast-mediated bone remodeling in turn releases bone matrix-associated TGF-β and other growth factors into the local environment, and TGF-β readily induces expression of IL-11 and other osteoclast differentiation factors in breast cancer cells thereby further increasing the rate of bone loss [91]. Accordingly, ablation of the TGF-β-signaling node SMAD4 attenuated the capacity of breast cancer cells to produce IL-11 and to metastasize to bone [95]. IL-11 over-expression alone failed to increase the rate by which MDA-MB-231 cells formed bone metastases, suggesting that IL-11 may not be involved in the homing of disseminated cancer cells to bone. In this model, however, ectopic IL-11 expression co-operated with over-expression of the chemokine receptor CXCR4 to drive experimental osteolytic metastasis [39].

Advanced colorectal cancer has a high propensity to metastasize to the liver *via* the portal vein. Primary colorectal tumors produce high levels of TGF-β [35], though unlike breast cancer cells they mostly lack the ability to respond to TGF-β signaling owing to frequent mutational inactivation of components of the TGF-β pathway [96]. Calon and colleagues identified a pro-metastatic pathway involving TGF-β mediated induction of IL-11 expression in the CAFs associated with primary tumors. They further proposed that paracrine stimulation of cancer cells with IL-11 activates STAT3 to facilitate metastatic colonization mainly through evasion of apoptosis, and this was inhibited by silencing GP130 expression on cancer cells. Although it was not formally proven that the colorectal cancer cells express IL-11Rα, it is reminiscent of findings demonstrating that the growth of primary colonic adenomas is impaired in bone marrow chimeras where the host, but not the bone marrow, lack IL-11Rα expression [22]. It remains unclear whether TGF-β can induce IL-11 expression in CAFs irrespective of the site of the emerging micrometastasis. Interestingly, while colorectal cancer cell lines engineered to produce IL-11 displayed enhanced spontaneous metastasis from the orthotopic site in the cecal wall to multiple organs, ectopic over-expression of IL-6 only marginally enhanced metastasis [35]. The latter is most likely attributable to the low abundance of IL-6Rα on these cells, and also suggests the absence of significant IL-6 *trans*-signaling, whereby cells lacking the trans-membrane IL-6Rα can respond to a preformed soluble complex comprising IL-6 and the cleaved form of the extracellular domain of IL-6Rα [97].

An alternative, and not mutually excluding mechanism suggests that TGF-β may also elevate IL-11 levels by stabilizing its mRNA through induction of the long non-coding RNA lncRNA-ATB in hepatocellular cancer cells. The latter appears to also sequester miR-200 family microRNAs [92]. Inhibition of miR-200 family miRNAs promoted epithelial-to-mesenchymal transition and cell invasion, while the increased IL-11 production resulted in autocrine STAT3 activation, although without modulating invasion. Similar to the aforementioned study from Calon et al., Yuan et al. [92] also observed that lncRNA-ATB expression in two different models of experimental metastasis, promoted tumor cell survival during the early phases of metastatic colonization though an IL-11/STAT3-dependent mechanism. Finally, IL-11 mRNA levels are significantly higher in portal vein tumor thrombi than in matched primary liver cancers from the same patient, which

coincides with the capacity of hepatocellular carcinomas to metastasize intra-hepatically *via* the portal vein.

Finally, it is worthwhile to consider that IL-11 may also promote metastasis indirectly owing to its capacity to stimulate platelet production [98,99]. Platelets can coalesce with circulating tumor cells in the bloodstream to both protect them from the immune system as well as assist cancer cell extravasation into tissue parenchyma [100].

5. Therapeutically targeting IL-11 signaling in cancer

To date, only a few studies have been published that document therapeutic targeting of IL-11 signaling in pre-clinical models of cancer. The viability and largely normal physiological response of adult *Il11ra*-null mice, with the lack of decidua formation in pregnant females being the most obvious defect [101], suggests that targeting of IL-11 signaling in cancer patients is likely to avoid major deleterious systemic side effects [102]. Likewise, prolonged treatment of mice with an antagonistic form of IL-11, designated mIL-11 Mutein and possessing over 20-fold higher affinity for mouse IL-11Rα than wild-type mIL-11 [103], did not result in a drop in blood platelets or affect blood coagulation [22].

IL-11, rather than IL-6, was shown to drive the growth of gastrointestinal cancers in mouse models [22]. In a genetically engineered model of inflammation-associated gastric cancer, IL-11 acted directly on tumor cells to drive STAT3 activation, cellular proliferation and invasion. Moreover, administration of mIL-11 Mutein reduced gastric tumor burden in these mice coinciding with reduced proliferation and enhanced apoptosis of tumor cells and a reduction in both tumor-associated inflammatory cells and cytokine levels [22]. Treatment with mIL-11 Mutein also reduced tumor size and multiplicity in a mouse model of carcinogen-induced sporadic colorectal cancer and attenuated growth of DLD-1 colorectal cancer xenografts [22]. Inhibition of tumor growth using this strategy is likely to require sustained administration of the therapeutic as the growth of gastric tumors rebounded following withdrawal of mIL-11 Mutein. It is possible that combination therapy using mIL-11 Mutein in conjunction with standard chemotherapy may result in complete tumor remissions, although this has not been formally evaluated experimentally.

Several antibodies that bind to and inhibit IL-11 [104], IL-11Rα (Putoczki T.L., unpublished observations) or GP130 have been developed and shown efficacy in various mouse models. Likewise, antibodies targeting human IL-11 (R&D Systems), IL-11Rα (Putoczki T.L., unpublished observations) or GP130 [105] are being developed for preclinical evaluation, on the back of the clinical success of antibodies targeting IL-6 (Siltuximab) or IL-6Rα (Tocilizumab). Indeed, targeting IL-11 or IL-11Rα may prevent unwanted side-effects arising from targeting GP130, given the embryonic lethality of *gp130* knockout mice [106], although GP130 epitopes have been identified that allowed for the development of GP130 antibodies that specifically inhibit IL-11 signaling [107]. The IL-11Rα has also been used to exploit chimeric antigen-receptor (CAR) strategies to mediate cytotoxic T-cell killing of a human osteosarcoma cell line [108].

Using *in vivo* phage display on patient-derived prostate cancer tissue, Pasqualini and colleagues identified peptides with ability to bind to tumor vascular endothelium [109,110]. One peptide bound to IL-11Rα expressed on endothelial cells [110] because it mimicked a motif in IL-11 that binds the receptor. Conjugation of this peptide motif to an apoptosis-inducing peptide sequence yielded a peptidomimetic, which targeted bone metastases and showed promising anti-tumor activity in pre-clinical models of prostate cancer and osteosarcoma [111,112]. While these studies used cell surface IL-11Rα as means for delivering therapeutics to tumors, they do not address the exact nature of the IL-11 responsive cell type(s) that serve(s) as the Achilles heel for the growth and survival of the tumors. However, these studies did suggest the involvement of IL-11 signaling in tumor angiogenesis and in light of the aforementioned insights from the study by Marusyk et al. [68], this is likely to be a mechanism by which inhibition of IL-11 signaling in breast cancer may confer clinical benefit.

A more generic approach of interfering with GP130-mediated signaling is afforded by the various small molecule kinase inhibitors currently being either in advanced clinical trial or following repurposing of the FDA-approved JAK2 inhibitors for the treatment myeloproliferative and other hematological diseases [113]. It is likely that development of additional novel JAK tyrosine kinase inhibitors that better distinguish between the four highly related family members are likely to reduce the dose-limiting toxicity, including thrombocytopenia that is often observed with the current compounds [114].

6. Conclusions

Analysis of the recent literature strongly indicates a complex multi-faceted pro-tumorigenic role for IL-11, including for breast cancer. IL-11 has now been implicated in several unrelated aspects of tumor biology. These include the promotion of angiogenesis, survival under hypoxic conditions, apoptosis and chemoresistance, as well as growth and survival of early micro-metastatic colonies in bone and soft tissues including liver and lung. Further investigations are required to pinpoint the precise mechanisms by which IL-11 confers each of these activities.

Based on the observations summarized here, blockade of IL-11 signaling either through targeting of the ligand or of its cognate receptor, and in a more general approach through one of the many small molecule JAK tyrosine kinase inhibitors currently in clinical trials, is likely to generate collateral interference with processes that govern tumor homeostasis and progression. Key to this will be the careful assessment of the effect of anti-IL-11 therapeutics on primary tumors and distant metastasis in well-characterized xenograft and syngeneic mouse models of breast cancer. Eventual translation of this approach to the clinical setting will require selection of patients most likely to benefit from anti-IL-11 therapy. Since IL-11 can promote cancer progression through both direct action on cancer cells and indirectly *via* effects on the tumor microenvironment, screening of patients for IL-11/IL-11Rα expression or other appropriate surrogate markers may need to be considered in the fullness of time.

Acknowledgments

State Government Operational Infrastructure Support, and NHMRC grants 1008614 and 1080498 (to T.L. Putoczki) and 603122, 1064987, 1069024 and 1079257 (to M. Ernst) and WCR, formally AICR (to T.L. Putoczki) and the WEHI Dyson Centenary Fellowship (to T.L. Putoczki). The laboratory of M. Ernst also receives financial supported from Ludwig Cancer Research. M. Ernst and T.L. Putoczki are inventors of patents describing the use of IL-11 antagonists for the treatment of cancer. The authors have no additional relevant existing or perceived financial interest.

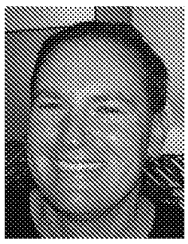
References

[1] M. Ernst, T.L. Putoczki, Molecular pathways: IL11 as a tumor-promoting cytokine-translational implications for cancers, Clin. Cancer Res. 20 (2014) 5579–5588.
[2] T.L. Putoczki, M. Ernst, IL-11 signaling as a therapeutic target for cancer, Immunotherapy 7 (2015) 441–453.
[3] J.S. Stumhofer, E.D. Tait, W.J. Quinn 3rd, N. Hosken, B. Spudy, R. Goenka, et al., A role for IL-27p28 as an antagonist of gp130-mediated signaling, Nat. Immunol. 11 (2010) 1119–1126.

[4] T. Taga, T. Kishimoto, Gp130 and the interleukin-6 family of cytokines, Annu. Rev. Immunol. 15 (1997) 797–819.
[5] S.R. Dillon, C. Sprecher, A. Hammond, J. Bilsborough, M. Rosenfeld-Franklin, S.R. Presnell, et al., Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice, Nat. Immunol. 5 (2004) 752–760.
[6] G.C. Elson, E. Lelievre, C. Guillet, S. Chevalier, H. Plun-Favreau, J. Froger, et al., CLF associates with CLC to form a functional heteromeric ligand for the CNTF receptor complex, Nat. Neurosci. 3 (2000) 867–872.
[7] D. Pennica, K.L. King, K.J. Shaw, E. Luis, J. Rullamas, S.M. Luoh, et al., Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 1142–1146.
[8] T.M. Rose, A.G. Bruce, Oncostatin M is a member of a cytokine family that includes leukemia-inhibitory factor, granulocyte colony-stimulating factor, and interleukin 6, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 8641–8645.
[9] D.P. Gearing, N.M. Gough, J.A. King, D.J. Hilton, N.A. Nicola, R.J. Simpson, et al., Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF), EMBO J. 6 (1987) 3995–4002.
[10] S.R. Paul, F. Bennett, J.A. Calvetti, K. Kelleher, C.R. Wood, R.M. O'Hara Jr., et al., Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymphopoietic and hematopoietic cytokine, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 7512–7516.
[11] J.S. Silver, C.A. Hunter, gp130 at the nexus of inflammation, autoimmunity, and cancer, J. Leukoc. Biol. 88 (2010) 1145–1156.
[12] M. Ernst, B.J. Jenkins, Acquiring signalling specificity from the cytokine receptor gp130, Trends Genet. 20 (2004) 23–32.
[13] T.L. Putoczki, K.C. Dobson, M.D. Griffin, The structure of human interleukin-11 reveals receptor-binding site features and structural differences from interleukin-6, Acta Crystallogr. D: Biol. Crystallogr. 70 (2014) 2277–2285.
[14] T. Yin, K. Yasukawa, T. Taga, T. Kishimoto, Y.C. Yang, Identification of a 130-kilodalton tyrosine-phosphorylated protein induced by interleukin-11 as JAK2 tyrosine kinase, which associates with gp130 signal transducer, Exp. Hematol. 22 (1994) 467–472.
[15] A. Orazi, X. Du, Z. Yang, M. Kashai, D.A. Williams, Interleukin-11 prevents apoptosis and accelerates recovery of small intestinal mucosa in mice treated with combined chemotherapy and radiation, Lab. Invest. 75 (1996) 33–42.
[16] N.S. Welch, A. Wang, M. Fitzgerald, T.Y. Neben, D. Donaldson, J. Giannotti, et al., Recombinant human interleukin-11 directly promotes megakaryocytopoiesis in vitro, Blood 90 (1997) 3893–3902.
[17] I. Tepler, L. Elias, J.W. Smith, 2nd, M. Hussein, G. Rosen, A.Y. Chang, et al., A randomized placebo-controlled trial of recombinant human interleukin-11 in cancer patients with severe thrombocytopenia due to chemotherapy, Blood 87 (1996) 3607–3614.
[18] M.S. Gordon, W.J. McCaskill-Stevens, L.A. Battiato, J. Loewy, D. Loesch, E. Breeden, et al., A phase I trial of recombinant human interleukin-11 (neumega rhIL-11 growth factor) in women with breast cancer receiving chemotherapy, Blood 87 (1996) 3615–3624.
[19] S. Wu, Y. Zhang, L. Xu, Y. Dai, Y. Teng, S. Ma, et al., Multicenter, randomized study of genetically modified recombinant human interleukin-11 to prevent chemotherapy-induced thrombocytopenia in cancer patients receiving chemotherapy, Support Care Cancer 20 (2012) 1875–1884.
[20] J.A. Kaye, Clinical development of recombinant human interleukin-11 to treat chemotherapy-induced thrombocytopenia, Curr. Opin. Hematol. 3 (1996) 209–215.
[21] L. Robb, R. Li, L. Hartley, H.H. Nandurkar, F. Koentgen, C.G. Begley, Infertility in female mice lacking the receptor for interleukin 11 is due to a defective uterine response to implantation, Nat. Med. 4 (1998) 303–308.
[22] T.L. Putoczki, S. Thiem, A. Loving, R.A. Busuttil, N.J. Wilson, P.K. Ziegler, et al., Interleukin-11 is the dominant IL-6 family cytokine during gastrointestinal tumorigenesis and can be targeted therapeutically, Cancer Cell 24 (2013) 257–271.
[23] L. Robb, D.J. Hilton, T.A. Willson, C.G. Begley, Structural analysis of the gene encoding the murine interleukin-11 receptor alpha-chain and a related locus, J. Biol. Chem. 271 (1996) 13754–13761.
[24] J. Grotzinger, T. Kernebeck, K.J. Kallen, S. Rose-John, IL-6 type cytokine receptor complexes: hexamer, tetramer or both? Biol. Chem. 380 (1999) 803–813.
[25] R. Matadeen, W.C. Hon, J.K. Heath, E.Y. Jones, S. Fuller, The dynamics of signal triggering in a gp130-receptor complex, Structure 15 (2007) 441–448.
[26] B.J. Jenkins, D. Grail, T. Nheu, M. Najdovska, B. Wang, P. Waring, et al., Hyperactivation of Stat3 in gp130 mutant mice promotes gastric hyperproliferation and desensitizes TGF-beta signaling, Nat. Med. 11 (2005) 845–852.
[27] D.J. Gough, A. Corlett, K. Schlessinger, J. Wegrzyn, A.C. Larner, D.E. Levy, Mitochondrial STAT3 supports Ras-dependent oncogenic transformation, Science 324 (2009) 1713–1716.
[28] A. Jarnicki, T. Putoczki, M. Ernst, Stat3: linking inflammation to epithelial cancer – more than a "gut" feeling? Cell Div. 5 (2010) 14.
[29] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674.
[30] J.J. Babon, L.N. Varghese, N.A. Nicola, Inhibition of IL-6 family cytokines by SOCS3, Semin. Immunol. 26 (2014) 13–19.
[31] M. Dance, A. Montagner, J.P. Salles, A. Yart, P. Raynal, The molecular functions of Shp2 in the Ras/mitogen-activated protein kinase (ERK1/2) pathway, Cell Signal. 20 (2008) 453–459.
[32] S. Thiem, T.P. Pierce, M. Palmieri, T.L. Putoczki, M. Buchert, A. Preaudet, et al., mTORC1 inhibition restricts inflammation-associated gastrointestinal tumorigenesis in mice, J. Clin. Invest. 123 (2013) 767–781.
[33] D.F. Quail, J.A. Joyce, Microenvironmental regulation of tumor progression and metastasis, Nat. Med. 19 (2013) 1423–1437.
[34] J.A. Joyce, J.W. Pollard, Microenvironmental regulation of metastasis, Nat. Rev. Cancer 9 (2009) 239–252.
[35] A. Calon, E. Espinet, S. Palomo-Ponce, D.V. Tauriello, M. Iglesias, M.V. Cespedes, et al., Dependency of colorectal cancer on a TGF-beta-driven program in stromal cells for metastasis initiation, Cancer Cell 22 (2012) 571–584.
[36] R.M. Neve, K. Chin, J. Fridlyand, J. Yeh, F.L. Baehner, T. Fevr, et al., A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes, Cancer Cell 10 (2006) 515–527.
[37] K.P. Hoeflich, C. O'Brien, Z. Boyd, G. Cavet, S. Guerrero, K. Jung, et al., In vivo antitumor activity of MEK and phosphatidylinositol 3-kinase inhibitors in basal-like breast cancer models, Clin. Cancer Res. 15 (2009) 4649–4664.
[38] A.M. Douglas, G.A. Goss, R.L. Sutherland, D.J. Hilton, M.C. Berndt, N.A. Nicola, et al., Expression and function of members of the cytokine receptor superfamily on breast cancer cells, Oncogene 14 (1997) 661–669.
[39] Y. Kang, P.M. Siegel, W. Shu, M. Drobnjak, S.M. Kakonen, C. Cordon-Cardo, et al., A multigenic program mediating breast cancer metastasis to bone, Cancer Cell 3 (2003) 537–549.
[40] M. Lacroix, B. Siwek, P.J. Marie, J.J. Body, Production and regulation of interleukin-11 by breast cancer cells, Cancer Lett. 127 (1998) 29–35.
[41] M.B. Crichton, J.E. Nichols, Y. Zhao, S.E. Bulun, E.R. Simpson, Expression of transcripts of interleukin-6 and related cytokines by human breast tumors, breast cancer cells, and adipose stromal cells, Mol. Cell. Endocrinol. 118 (1996) 215–220.
[42] D.R. Rhodes, S. Kalyana-Sundaram, V. Mahavisno, R. Varambally, J. Yu, B.B. Briggs, et al., Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles, Neoplasia 9 (2007) 166–180.
[43] Cancer Genome Atlas N, Comprehensive molecular portraits of human breast tumours, Nature 490 (2012) 61–70.
[44] C. Curtis, S.P. Shah, S.F. Chin, G. Turashvili, O.M. Rueda, M.J. Dunning, et al., The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups, Nature 486 (2012) 346–352.
[45] A.J. Davidson, S.A. Freeman, K.E. Crosier, C.R. Wood, P.S. Crosier, Expression of murine interleukin 11 and its receptor alpha-chain in adult and embryonic tissues, Stem Cells 15 (1997) 119–124.
[46] B. Singh, J.A. Berry, A. Shoher, A. Lucci, COX-2 induces IL-11 production in human breast cancer cells, J. Surg. Res. 131 (2006) 267–275.
[47] X.Z. Shi, S.K. Sarna, Transcriptional regulation of inflammatory mediators secreted by human colonic circular smooth muscle cells, Am. J. Physiol. Gastrointest. Liver Physiol. 289 (2005) G274–G284.
[48] E. Stickeler, D. Pils, M. Klar, M. Orlowski-Volk, A. Zur Hausen, M. Jager, et al., Basal-like molecular subtype and HER4 up-regulation and response to neoadjuvant chemotherapy in breast cancer, Oncol. Rep. 26 (2011) 1037–1045.
[49] M.J. Magbanua, D.M. Wolf, C. Yau, S.E. Davis, J. Crothers, A. Au, et al., Serial expression analysis of breast tumors during neoadjuvant chemotherapy reveals changes in cell cycle and immune pathways associated with recurrence and response, Breast Cancer Res. 17 (2015) 73.
[50] L.A. Gilbert, M.T. Hemann, DNA damage-mediated induction of a chemoresistant niche, Cell 143 (2010) 355–366.
[51] M. Ernst, M. Najdovska, D. Grail, T. Lundgren-May, M. Buchert, H. Tye, et al., STAT3 and STAT1 mediate IL-11-dependent and inflammation-associated gastric tumorigenesis in gp130 receptor mutant mice, J. Clin. Invest. 118 (2008) 1727–1738.
[52] S. Hanavadi, T.A. Martin, G. Watkins, R.E. Mansel, W.G. Jiang, Expression of interleukin 11 and its receptor and their prognostic value in human breast cancer, Ann. Surg. Oncol. 13 (2006) 802–808.
[53] B.D. Lehmann, J.A. Bauer, X. Chen, M.E. Sanders, A.B. Chakravarthy, Y. Shyr, et al., Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies, J. Clin. Invest. 121 (2011) 2750–2767.
[54] K.D. Yu, R. Zhu, M. Zhan, A.A. Rodriguez, W. Yang, S. Wong, et al., Identification of prognosis-relevant subgroups in patients with chemoresistant triple-negative breast cancer, Clin. Cancer Res. 19 (2013) 2723–2733.
[55] J. Bockhorn, R. Dalton, C. Nwachukwu, S. Huang, A. Prat, K. Yee, et al., MicroRNA-30c inhibits human breast tumour chemotherapy resistance by regulating TWF1 and IL-11, Nat. Commun. 4 (2013) 1393.
[56] G. Kim, M. Ouzounova, A.A. Quraishi, A. Davis, N. Tawakkol, S.G. Clouthier, et al., SOCS3-mediated regulation of inflammatory cytokines in PTEN and p53 inactivated triple negative breast cancer model, Oncogene 34 (2015) 671–680.
[57] Z.L. Xiang, Z.C. Zeng, J. Fan, Z.Y. Tang, H.Y. Zeng, Expression of connective tissue growth factor and interleukin-11 in intratumoral tissue is associated with poor survival after curative resection of hepatocellular carcinoma, Mol. Biol. Rep. 39 (2012) 6001–6006.
[58] L.G. Necula, M. Chivu-Economescu, E.L. Stanciulescu, C. Bleotu, S.O. Dima, I. Alexiu, et al., IL-6 and IL-11 as markers for tumor aggressiveness and prognosis in gastric adenocarcinoma patients without mutations in Gp130 subunits, J. Gastrointest. Liver Dis. 21 (2012) 23–29.

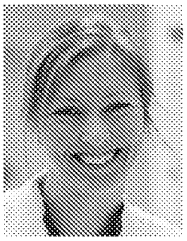
[59] D. Pan, L. Xu, H. Liu, W. Zhang, W. Liu, Y. Liu, et al., High expression of interleukin-11 is an independent indicator of poor prognosis in clear-cell renal cell carcinoma, Cancer Sci. 106 (2015) 592–597.
[60] B. Gyorffy, P. Surowiak, J. Budczies, A. Lanczky, Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer, PLOS ONE 8 (2013) e82241.
[61] C. Sotiriou, M. Lacroix, L. Lespagnard, D. Larsimont, M. Paesmans, J.J. Body, Interleukins-6 and -11 expression in primary breast cancer and subsequent development of bone metastases, Cancer Lett. 169 (2001) 87–95.
[62] L. Ren, X. Wang, Z. Dong, J. Liu, S. Zhang, Bone metastasis from breast cancer involves elevated IL-11 expression and the gp130/STAT3 pathway, Med. Oncol. 30 (2013) 634.
[63] C. Desmedt, F. Piette, S. Loi, Y. Wang, F. Lallemand, B. Haibe-Kains, et al., Strong time dependence of the 76-gene prognostic signature for node-negative breast cancer patients in the TRANSBIG multicenter independent validation series, Clin. Cancer Res. 13 (2007) 3207–3214.
[64] S.F. Madden, C. Clarke, P. Gaule, S.T. Aherne, N. O'Donovan, M. Clynes, et al., BreastMark: an integrated approach to mining publicly available transcriptomic datasets relating to breast cancer outcome, Breast Cancer Res. 15 (2013) R52.
[65] J. Folkman, Tumor angiogenesis: therapeutic implications, N. Engl. J. Med. 285 (1971) 1182–1186.
[66] J.E. Jung, H.G. Lee, I.H. Cho, D.H. Chung, S.H. Yoon, Y.M. Yang, et al., STAT3 is a potential modulator of HIF-1-mediated VEGF expression in human renal carcinoma cells, FASEB J. 19 (2005) 1296–1298.
[67] B. Onnis, N. Fer, A. Rapisarda, V.S. Perez, G. Melillo, Autocrine production of IL-11 mediates tumorigenicity in hypoxic cancer cells, J. Clin. Invest. 123 (2013) 1615–1629.
[68] A. Marusyk, D.P. Tabassum, P.M. Altrock, V. Almendro, F. Michor, K. Polyak, Non-cell-autonomous driving of tumour growth supports sub-clonal heterogeneity, Nature 514 (2014) 54–58.
[69] K. Mahboubi, E.C. Biedermann, J.M. Carroll, J.S. Pober, IL-11 activates human endothelial cells to resist immune-mediated injury, J. Immunol. 164 (2000) 3837–3846.
[70] K. Pardal, M.F. Clarke, S.J. Morrison, Applying the principles of stem-cell biology to cancer, Nat. Rev. Cancer 3 (2003) 895–902.
[71] M. Al-Hajj, M.S. Wicha, A. Benito-Hernandez, S.J. Morrison, M.F. Clarke, Prospective identification of tumorigenic breast cancer cells, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 3983–3988.
[72] C. Ginestier, M.H. Hur, E. Charafe-Jauffret, F. Monville, J. Dutcher, M. Brown, et al., ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome, Cell Stem Cell 1 (2007) 555–567.
[73] C. Scheel, E.N. Eaton, S.H. Li, C.L. Chaffer, F. Reinhardt, K.J. Kah, et al., Paracrine and autocrine signals induce and maintain mesenchymal and stem cell states in the breast, Cell 145 (2011) 926–940.
[74] C.L. Chaffer, I. Brueckmann, C. Scheel, A.J. Kaestli, P.A. Wiggins, L.O. Rodrigues, et al., Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 7950–7955.
[75] D. Iliopoulos, H.A. Hirsch, G. Wang, K. Struhl, Inducible formation of breast cancer stem cells and their dynamic equilibrium with non-stem cancer cells via IL6 secretion, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 1397–1402.
[76] H. Yu, D. Pardoll, R. Jove, STATs in cancer inflammation and immunity: a leading role for STAT3, Nat. Rev. Cancer 9 (2009) 798–809.
[77] L. Lin, B. Hutzen, H.F. Lee, Z. Peng, W. Wang, C. Zhao, et al., Evaluation of STAT3 signaling in ALDH+ and ALDH+/CD44+/CD24− subpopulations of breast cancer cells, PLOS ONE 8 (2013) e82821.
[78] P. Sansone, G. Storci, S. Tavolari, T. Guarnieri, C. Giovannini, M. Taffurelli, et al., IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland, J. Clin. Invest. 117 (2007) 3988–4002.
[79] L.L. Marotta, V. Almendro, A. Marusyk, M. Shipitsin, J. Schemme, S.R. Walker, et al., The JAK2/STAT3 signaling pathway is required for growth of CD44(+)CD24(−) stem cell-like breast cancer cells in human tumors, J. Clin. Invest. 121 (2011) 2723–2735.
[80] K. Berns, H.M. Horlings, B.T. Hennessy, M. Madiredjo, E.M. Hijmans, K. Beelen, et al., A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer, Cancer Cell 12 (2007) 395–402.
[81] H. Korkaya, G.I. Kim, A. Davis, F. Malik, N.I. Henry, S. Ithimakin, et al., Activation of an IL6 inflammatory loop mediates trastuzumab resistance in HER2+ breast cancer by expanding the cancer stem cell population, Mol. Cell 47 (2012) 570–584.
[82] F.C. Adkins, A.M. Gonzalez-Angulo, X. Lei, L.F. Hernandez-Aya, E.A. Mittendorf, J.K. Litton, et al., Triple-negative breast cancer is not a contraindication for breast conservation, Ann. Surg. Oncol. 18 (2011) 3164–3173.
[83] E. Simpson, G. Rubin, C. Clyne, K. Robertson, L. O'Donnell, M. Jones, et al., The role of local estrogen biosynthesis in males and females, Trends Endocrinol. Metab. 11 (2000) 184–188.
[84] Y. Miki, T. Suzuki, C. Tazawa, Y. Yamaguchi, K. Kitada, S. Honma, et al., Aromatase localization in human breast cancer tissues: possible interactions between intratumoral stromal and parenchymal cells, Cancer Res. 67 (2007) 3945–3954.
[85] V.R. Agarwal, S.E. Bulun, M. Leitch, R. Rohrich, S.R. Simpson, Use of alternative promoters to express the aromatase cytochrome P450 (CYP19) gene in breast adipose tissues of cancer-free and breast cancer patients, J. Clin. Endocrinol. Metab. 81 (1996) 3843–3849.
[86] S.Q. Yo, K.C. Knower, V. Cheung, E.R. Simpson, C.D. Clyne, Transcriptional control of local estrogen formation by aromatase in the breast, J. Steroid Biochem. Mol. Biol. 145 (2015) 179–186.
[87] E.R. Simpson, M.D. Michael, V.R. Agarwal, M.M. Hinshelwood, S.E. Bulun, Y. Zhao, Cytochromes P450 11: expression of the CYP19 (aromatase) gene: an unusual case of alternative promoter usage, FASEB J. 11 (1997) 29–36.
[88] F. De Miguel, S.O. Lee, S.A. Onate, A.C. Gao, Stat3 enhances transactivation of steroid hormone receptors, Nucl. Recept. 1 (2003) 3.
[89] Z. Zhang, S. Jones, J.S. Hagood, N.L. Fuentes, G.M. Fuller, STAT3 acts as a co-activator of glucocorticoid receptor signaling, J. Biol. Chem. 272 (1997) 30607–30610.
[90] P.G. Morris, C.A. Hudis, D. Giri, M. Morrow, D.J. Falcone, X.K. Zhou, et al., Inflammation and increased aromatase expression occur in the breast tissue of obese women with breast cancer, Cancer Prev. Res. 4 (2011) 1021–1029.
[91] Y. Kang, W. He, S. Tulley, G.P. Gupta, J. Serganova, C.R. Chen, et al., Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 13909–13914.
[92] J.H. Yuan, F. Yang, F. Wang, J.Z. Ma, Y.J. Guo, Q.F. Tao, et al., A long noncoding RNA activated by TGF-beta promotes the invasion-metastasis cascade in hepatocellular carcinoma, Cancer Cell 25 (2014) 666–681.
[93] G. Girasole, G. Passeri, R.L. Jilka, S.C. Manolagas, Interleukin-11: a new cytokine critical for osteoclast development, J. Clin. Invest. 93 (1994) 1516–1524.
[94] W. Kozlow, T.A. Guise, Breast cancer metastasis to bone: mechanisms of osteolysis and implications for therapy, J Mammary Gland Biol. Neoplasia 10 (2005) 169–180.
[95] M. Deckers, M. van Dinther, J. Buijs, I. Que, C. Lowik, G. van der Pluijm, et al., The tumor suppressor Smad4 is required for transforming growth factor beta-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells, Cancer Res. 66 (2006) 2202–2209.
[96] N. Bellam, B. Pasche, Tgf-beta signaling alterations and colon cancer, Cancer Treat. Res. 155 (2010) 85–103.
[97] S. Rose-John, IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6, Int. J. Biol. Sci. 8 (2012) 1237–1247.
[98] M. Musashi, Y.C. Yang, S.R. Paul, S.C. Clark, T. Sudo, M. Ogawa, Direct and synergistic effects of interleukin 11 on murine hemopoiesis in culture, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 765–769.
[99] S. Kobayashi, M. Teramura, I. Sugawara, K. Oshimi, H. Mizoguchi, Interleukin-11 acts as an autocrine growth factor for human megakaryoblastic cell lines, Blood 81 (1993) 889–893.
[100] L.J. Gay, B. Felding-Habermann, Contribution of platelets to tumour metastasis, Nat. Rev. Cancer 11 (2011) 123–134.
[101] P. Bilinski, D. Roopenian, A. Gossler, Maternal IL-11Ralpha function is required for normal decidua and fetoplacental development in mice, Genes Dev. 12 (1998) 2234–2243.
[102] H.H. Nandurkar, L. Robb, D. Tarlinton, L. Barnett, F. Kontgen, C.G. Begley, Adult mice with targeted mutation of the interleukin-11 receptor (IL11Ra) display normal hematopoiesis, Blood 90 (1997) 2148–2159.
[103] C.G. Lee, D. Hartl, H. Matsuura, F.M. Dunlop, P.D. Scouney, L.J. Fabri, et al., Endogenous IL-11 signaling is essential in Th2- and IL-13-induced inflammation and mucus production, Am. J. Respir. Cell. Mol. Biol. 39 (2008) 739–746.
[104] M.A. Kapina, G.S. Shepelkova, V.G. Avdeenko, A.N. Guseva, T.K. Kondratieva, V.V. Evstifeev, et al., Interleukin-11 drives early lung inflammation during Mycobacterium tuberculosis infection in genetically susceptible mice, PLoS ONE 6 (2011) e21878.
[105] S. Xu, N. Neamati, gp130: a promising drug target for cancer therapy, Expert Opin. Ther. Targets 17 (2013) 1303–1328.
[106] K. Yoshida, T. Taga, M. Saito, S. Suematsu, A. Kumanogoh, T. Tanaka, et al., Targeted disruption of gp130, a common signal transducer for the interleukin 6 family of cytokines, leads to myocardial and hematological disorders, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 407–411.
[107] J. Sommer, T. Effenberger, E. Volpi, G.H. Waetzig, M. Bernhardt, J. Suthaus, et al., Constitutively active mutant gp130 receptor protein from inflammatory hepatocellular adenoma is inhibited by an anti-gp130 antibody that specifically neutralizes interleukin 11 signaling, J. Biol. Chem. 287 (2012) 13743–13751.
[108] G. Huang, L. Yu, L.J. Cooper, M. Hollomon, H. Huls, E.S. Kleinerman, Genetically modified T cells targeting interleukin-11 receptor alpha-chain kill human osteosarcoma cells and induce the regression of established osteosarcoma lung metastases, Cancer Res. 72 (2012) 271–281.
[109] W. Arap, M.G. Kolonin, M. Trepel, J. Lahdenranta, M. Cardo-Vila, R.J. Giordano, et al., Steps toward mapping the human vasculature by phage display, Nat. Med. 8 (2002) 121–127.
[110] A.J. Zurita, P. Troncoso, M. Cardo-Vila, C.J. Logothetis, R. Pasqualini, W. Arap, Combinatorial screenings in patients: the interleukin-11 receptor alpha as a candidate target in the progression of human prostate cancer, Cancer Res. 64 (2004) 435–439.

[111] R. Pasqualini, R.E. Millikan, D.R. Christianson, M. Cardo-Vila, W.H. Driessen, R.J. Giordano, et al., Targeting the interleukin-11 receptor alpha in metastatic prostate cancer: a first-in-man study, Cancer 121 (2015) 2411–2421.
[112] V.O. Lewis, M.G. Ozawa, M.T. Deavers, G. Wang, T. Shintani, W. Arap, et al., The interleukin-11 receptor alpha as a candidate ligand-directed target in osteosarcoma: consistent data from cell lines, orthotopic models, and human tumor samples, Cancer Res. 69 (2009) 1995–1999.
[113] M. Buchert, C.J. Burns, M. Ernst, Targeting JAK kinase in solid tumors: emerging opportunities and challenges, Oncogene (2015), http://dx.doi.org/10.1038/onc.2015.150, May 18 [Epub ahead of print] Review. PMID: 25982279.
[114] A. Tefferi, JAK inhibitors for myeloproliferative neoplasms: clarifying facts from myths, Blood 119 (2012) 2721–2730.
[115] R.K. Albert, J. Connett, W.C. Bailey, R. Casaburi, J.A. Cooper Jr., G.J. Criner, et al., Azithromycin for prevention of exacerbations of COPD, N. Engl. J. Med. 365 (2011) 689–698.
[116] A. Abdollahi, P. Hahnfeldt, C. Maercker, H.J. Grone, J. Debus, W. Ansorge, et al., Endostatin's antiangiogenic signaling network, Mol. Cell 13 (2004) 649–663.
[117] A. Prat, J.S. Parker, O. Karginova, C. Fan, C. Livasy, J.I. Herschkowitz, et al., Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer, Breast Cancer Res. 12 (2010) R68.
[118] F.M. Buffa, C. Camps, L. Winchester, C.E. Snell, H.E. Gee, H. Sheldon, et al., microRNA-associated progression pathways and potential therapeutic targets identified by integrated mRNA and microRNA expression profiling in breast cancer, Cancer Res. 71 (2011) 5635–5645.
[119] S. Gluck, J.S. Ross, M. Royce, E.F. McKenna Jr., C.M. Perou, E. Avisar, et al., TP53 genomics predict higher clinical and pathologic tumor response in operable early-stage breast cancer treated with docetaxel-capecitabine +/- trastuzumab, Breast Cancer Res. Treat. 132 (2012) 781–791.
[120] G. Finak, N. Bertos, F. Pepin, S. Sadekova, M. Souleimanova, H. Zhao, et al., Stromal gene expression predicts clinical outcome in breast cancer, Nat. Med. 14 (2008) 518–527.



Dr. Cameron Johnstone is a post-doctoral fellow and received his Ph.D. from the Ludwig Institute for Cancer Research, Melbourne in 2000 where he investigated the regulation of intestine-specific expression of the gene encoding the transmembrane glycoprotein A33. He received his post-doctoral training in the laboratory of Prof Anil Rustgi, at the University of Pennsylvania investigating the functions of protein- (ARHGAP8, PARVB, PRR5) and microRNA-encoding genes (Let-7a, Let-7b) on chromosome 22q in the pathogenesis of breast and colorectal cancer. Returning to Melbourne in 2009, he commenced at the Peter MacCallum Cancer Centre to investigate protein-encoding and microRNA genes in breast cancer invasion, progression and metastasis using novel syngeneic and xenograft mouse models. He joined the Olivia Newton John Cancer Research Institute in 2015 to further elucidate the roles of IL-11 in breast cancer oncogenesis.

Dr. Ashwini Chand holds a Bachelors and Masters degree in biochemistry from the University of Otago, Dunedin, New Zealand. Her Ph.D. research was conducted at the University of Auckland, which she completed in 2006. She is a Senior Research Officer in the Cancer Inflammation Laboratory and currently holds the NHMRC Career Development Fellowship. Dr Chand's work is focused on the study of tumor and stroma interactions that mediate breast cancer initiation and progression. Projects Dr Chand is currently working on aim to identify novel nuclear receptor-associated transcriptional networks, as well as understanding cell-signaling pathways within the tumor microenvironment.



Dr. Putoczki completed her undergraduate research training in 2002 with the Departments of Medical Biophysics and Anatomy at the University of Toronto, Canada. In 2006, she received her Ph.D. in Cellular and Molecular Biology from the University of Canterbury, in Christchurch Zealand. During her postdoctoral training at the Ludwig Institute for Cancer Research, in Melbourne she developed an intense interest in the contribution of cytokines to gastrointestinal tumor formation. Dr Putoczki is now a laboratory head in the Inflammation Division at the Walter and Eliza Hall Institute in Melbourne. Her research team studies the role of the microenvironment in the progression of gastrointestinal disease, with the aim of identifying new therapeutic opportunities. The main focus of her laboratory is to define how signal transduction by cytokines can modulate tissue homeostasis, and to understand how deregulation of these signaling pathways can promote the initiation and spread of gastrointestinal cancer.

Prof. Matthias Ernst obtained his Ph.D. in Molecular Biology from the Swiss Institute of Technology (ETH) in Zurich (Switzerland). After postdocs in the *Merck Sharp & Dohme* (USA) and *Ludwig Institute for Cancer Research* in Melbourne, he became a group leader before moving to *Walter and Eliza Hall Institute*. He currently serves as the Scientific Director of the newly established *Olivia Newton-John Cancer Research Institute* in Melbourne. His laboratory embracing genetic tools to understand the molecular mechanism that regulate the cells of the lining of the gastrointestinal tract to continuously renew without forming tumors. His approach has defined novel therapeutic targets and enjoyed uninterrupted funding from the Commonwealth's National Health and Medical Research Council.

British Journal of Haematology, 1995, 91, 319–326

Interleukin-11 (IL-11) acts as a synergistic factor for the proliferation of human myeloid leukaemic cells

ROBERTO M. LEMOLI, MIRIAM FOGLI, ALESSANDRA FORTUNA, MARILINA AMABILE, PATRIZIA ZUCCHINI,* ALEXIS GRANDE,* GIOVANNI MARTINELLI, GIUSEPPE VISANI, SERGIO FERRARI* AND SANTE TURA
*Institute of Haematology, University of Bologna, and *Institute of Biological Chemistry, University of Modena, Italy*

Received 10 April 1995; accepted for publication 22 June 1995

Summary. Interleukin-11 is a stromal cells derived cytokine which stimulates the proliferation of primitive haemopoietic progenitor cells. For this paper we have studied the constitutive expression of IL-11 mRNA in a panel of well-known leukaemic cell lines and samples from AML patients at diagnosis. Moreover, the same cellular populations were evaluated for their proliferative response to recombinant-human-(r-hu) IL-11 alone and combined with r-hu-IL-3, granulocyte-macrophage colony stimulating factor (GM-CSF) and stem cell factor (SCF, c-kit ligand). The colony-forming ability of HL60, K562, KG1 cells and eight fresh AML cell populations was assessed by a clonogenic assay in methylcellulose. In eight additional AML cases the number of S-phase leukaemic cells induced by IL-11 was determined by the bromodeoxyuridine (BRDU) incorporation assay after 3 d of liquid culture.

IL-11, as single cytokine, did not stimulate the colony formation of the three myeloid cell lines under serum-containing and serum-free conditions. In contrast, the proliferation of the leukaemic cells in response to IL-3, GM-CSF and SCF was enhanced by co-incubation with IL-11, and this effect was reversed in blocking experiments by the anti-IL-11 Moab. When tested on primary AML samples, IL-11 alone showed little, if any, proliferative activity. However, it increased the IL-3-dependent blast colony formation in eight out of eight cases and GM-CSF in seven cases. IL-11 also augmented synergistically the number of CFU-L stimulated by SCF in seven cases. A combination of three factors (IL-11, SCF and IL-3) yielded optimal colony formation. The BRDU studies showed the significant increase of AML cells in S-phase when IL-11 was combined with SCF, whereas the two CSF had no activity on their own. Positive interaction was also observed when IL-11 was added to IL-3 supplemented cultures in five out of eight cases tested. Reverse transcriptase-polymerase chain reaction amplification (RT-PCR) demonstrated the constitutive expression of IL-11 mRNA in all the cell lines and 11/12 AML samples studied at diagnosis.

These results indicate that IL-11 is expressed in leukaemic myeloid cells and that their proliferation is regulated by the cytokine which acts as a synergistic factor.

Keywords: IL-11, leukaemic cells, cytokine expression, growth regulation.

IL-11 is a novel cytokine which was originally isolated from the primate stromal cell line PU-34 and subsequently from the human MRC 5 cell line (Paul *et al*, 1990). Biological characterization has demonstrated that IL-11 has pleiotropic effects on both murine and human haemopoiesis (Du & Williams, 1994). Its activity appears to be similar to that of IL-6 in enhancing IL-3-dependent megakaryocytopoiesis (Bruno *et al*, 1991; Yonemura *et al*, 1992), production of hepatic acute-phase protein (Braumann & Schendel, 1991) and recruitment of early murine and human haemopoietic progenitor cells in cell cycle by shortening their G0 phase (Musashi *et al*, 1991; Leary *et al*, 1992). More recent data suggests that IL-11 synergizes with intermediate-late-acting CSFs (i.e. IL-3 and GM-CSF) and SCF for promoting the proliferation of very primitive multipotential haemopoietic precursors (identified phenotypically as CD 34^+ $CD33^-$ DR^- cells) as well as more mature lineage-restricted progenitors (CD 34^+ $CD33^+$ DR^+ cells) (Lemoli *et al*, 1993; Keller *et al*, 1993). *In-vivo*, IL-11 stimulates platelet production in sublethally irradiated mice and accelerates neutrophil and platelet recovery in transplanted mice, especially if associated with SCF (Du *et al*, 1993a, b).

Early studies have also provided evidence that IL-11 may be involved in the regulation of myeloid leukaemic cell growth. This cytokine has been proved to stimulate the

Correspondence: Dr Roberto M. Lemoli, Institute of Haematology, 'L & A. Seràgnoli,' Via Massarenti 9, 40138 Bologna, Italy.

proliferation of two megakaryoblastic cell lines and its activity was inhibited by anti-IL-11 antibodies and IL-11 antisense oligonucleotides, suggesting that an autocrine growth loop may be operative in these cell lines (Kobayashi *et al*, 1993). Moreover, IL-11 has been shown to enhance CSF-dependent proliferation of AML cells (Hu *et al*, 1993).

To define further the role of IL-11 in the regulation of leukaemic cell proliferation we have evaluated the constitutive expression of IL-11 mRNA in a panel of human myeloid cell lines and primary samples from AML patients. Furthermore, we have studied the proliferative response of the same cellular populations to exogenous IL-11, alone and combined with SCF, IL-3 and GM-CSF. The results presented here indicate that IL-11 transcript is detectable in AML cells, and they provide further evidence that this cytokine may play a role in leukaemic cell development.

MATERIALS AND METHODS

Recombinant haemopoietic CSFs. Four human recombinant growth factors were used in this study: IL-11 and IL-3 were supplied by Genetics Institute (Cambridge, Mass., U.S.A.) and were used at the concentration of 10 U/ml and 50 ng/ml, respectively. IL-11 has a specific activity of $2{\cdot}44 \times 10^6$ U/mg. SCF and GM-CSF were provided by Amgen (Thousand Oaks, Calif., U.S.A.) and were added to the cultures at the concentrations of 100 ng/ml and 1000 U/ml. The concentration of IL-11 was chosen based on our previous experience (Lemoli *et al*, 1993). In addition, a dose–response curve applied to the cell lines did not show any difference in colony growth in the range of 1–100 U/ml of IL-11. Stock solutions of the CSFs were stored at −80°C whereas dilution vials were left at −20°C (CSFs were diluted in Iscove's Modified Dulbecco's Medium, IMDM, with 2% of Fetal Calf FCS Serum Sera Lab, Crawley Down, Sussex).

Leukaemic cell lines. Three human myeloid cell lines maintained in exponential growth conditions were studied. HL60 is a human acute promyelocytic leukaemia cell line growing with a doubling time of 24 h. K562 cells derive from a chronic myelogenous leukaemia (CML) blastic crisis with a doubling time of 20 h. The KG1 myeloblastic leukaemic cell line grows with a doubling time of 36 h. In addition, the myeloid cell line U937 was evaluated for the presence of IL-11 mRNA. These cell lines were cultured in IMDM supplemented with 10% FCS, 1% antibiotics and 1% l-glutamine at 37°C in a fully humidified atmosphere of 5% CO_2 air. Cell viability was always > 90% at the time of study. All the cell lines were free of *Mycoplasma* contamination.

AML samples. Cells were obtained from the peripheral blood (PB) or the bone marrow (BM) of 16 AML patients (Table I). The diagnosis of AML was established by morphologic criteria, by cytochemical staining and by surface-marker analysis using a panel of monoclonal antibodies (MoAbs). Leukaemic specimens were subclassified according to the FAB classification system (Bennett *et al*, 1985). The mononuclear cell (MNC) fraction was collected and cryopreserved as previously described (Lemoli *et al*, 1991). After thawing, viable cells were recovered by Ficoll-Hypaque gradient. 14/16 samples contained > 90% blast cells (Table I). The percentage of residual T cells was assessed by immunofluorescent staining with anti-T3 and T11 MoAbs and was, for all the patients, < 1%.

Table I. Clinical characteristics of the AML cases studied.

Patient	Age/sex	FAB classification	Blasts* (%)	Source of cells†
1	22/M	M2	95	PB
2	34/F	M4	98	PB
3	40/M	M2	50	PB
4	67/F	M1	92	PB
5	54/M	M4	94	BM
6	57/M	M4	90	PB
7	28/M	M5b	96	PB
8	32/F	M1	95	BM
9	17/F	M2	98	PB
10	29/F	M5a	84	PB
11	18/F	M5a	93	BM
12	52/M	M5a	94	BM
13	82/F	M4	91	PB
14	28/F	M5b	92	BM
15	54M	M4	94	BM
16	67F	M1	92	PB

*Percentages of blast cells were obtained by counting 200 cells from May-Grunwald-Giemsa stained cytocentrifuge smears prepared from thawed light-density MNC fractions

†PB: peripheral blood; BM: bone marrow.

Clonogenic assay. CFU-L were assayed as previously described (Lemoli *et al*, 1991). Briefly, the culture medium consisted of IMDM supplemented with 24% FCS, 0·8% bovine serum albumin (BSA) (Sigma Chemicals, St Louis, Mo., U.S.A.), 10^{-4} M of 2-mercaptoethanol (Sigma) and methylcellulose at a final concentration of 1%. To measure the optimum cloning efficiency, 20% of PHA-LCM was added to leukaemic samples. The number of cells plated was adjusted to have about 100 colonies per plate. Quadruplicate cultures were incubated at 37°C in 5% CO_2 air and colonies (> 20 cells) were scored after 10–14 d. The clonogenic assay for myeloid cell lines was the same as that used for CFU-L without the addition of PHA-LCM. Colonies (> 50 cells) were recorded after 10–14 d of culture. The clonogenic efficiency of HL60, K562 and KG1 was 25%, 12% and 18%, respectively.

Where indicated, FCS was replaced by a combination of 1% deionized-crystallized BSA (Sigma), 57 μg/ml of iron-saturated human transferrin (Sigma), 4·2 μg/ml of sodium selenite (Sigma) and 4·8 mg/ml of lecithin (Sigma) (serum-free cultures) (Lemoli *et al*, 1993).

Blocking experiments were performed by growing the leukaemic cells with and without 10 μg/ml of a monoclonal antibody directed to IL-11 (kind gift of Dr E. M. Alderman, Genetics Institute).

BRDU incorporation assay. The percentage of AML cells in S-phase was determined by the BRDU incorporation assay as described elsewhere (Lemoli *et al*, 1994). In brief, leukaemic

cells were seeded at 5×10^5 cells/ml in liquid culture of serum-containing IMDM. Cultures were maintained for 3 d in the presence of the different growth factors as indicated. At the time of analysis, 10 μl of the stock BRDU solution (1 mM) were added to the cell suspension for 30 min in 5% CO_2 at 37°C. After two washes, slides were obtained by cytocentrifugation, air-dried, fixed in a mixture of methanol and acetic acid (3 : 1), and denaturated in 0·07 M NaOH for 12 s. For nuclear BRDU staining, the slides were first incubated with 5 μl of anti-BRDU MoAb (Becton Dickinson), diluted 1 : 15 with PBS with 0·5% Tween 20 (Sigma) for 30 min at room temperature. Finally, they were treated with TRITC-conjugated anti-mouse Ig (Dako) diluted 1 : 30. Preparations were then washed overnight with PBS and positive cells were scored with a fluorescence microscope.

RNA purification. Total cellular RNA was extracted using a modification of the guanidinium–cesium chloride centrifugation technique (McDonald *et al*, 1987). The CsCl extraction method is characterized by a high degree of RNA purification. Furthermore, to avoid a potential genomic DNA contamination of purified RNA, all the samples were digested with RQ1-DNAse for 30 min at 37°C by adding 10 U RQ1 (Promega, Madison, Wis.), 40 U RNAsin (Promega), 1 × RQ1 buffer to a final volume of 200 μl. RNA concentration was then evaluated by reading the O.D. at 260 nm and by loading the samples on a 1% denaturing agarose gel. These quantitative controls are crucial to reverse transcribe the same amount of RNA from the different samples studied.

Oligonucleotide primers and probes and cDNA fragments labelling. Oligonucleotides primers and probes were synthesized with an automated solid-phase DNA synthesizer (Applied Biosystems Inc., Model 381A) with the standard fosforamidites chemistry and purified by several extractions with NH_4OH, incubated at 56°C for 16 h and ethanol precipitated or purified by PAGE. All the synthesized oligomers were previously compared with the gene bank DNAsis (Hitachi, Brisbaine, Calif.) in order to avoid homologies with other gene sequences. Furthermore, we synthesized oligonucleotide primers from separate exons for excluding a potential genomic DNA contamination of the RNA samples. The sequences of the cytokine primers and probes used in this study are: IL-11 direct primer (DP) 5′-CACATGAACTGTGTTTGCCGCCTGGT-3′ region 63–88; IL-11 reverse primer (RP) 5′-GCAGCCTTGTCAGCACACCTGG-GAGCTGTAGA-3′ region 326–357; IL-11 reverse probe (RP) 5′-TTTGTCCCTCAGCTGTGCAGCCAGCTGCCG-3′ region 225–254. Oligonucleotide probes and cDNA fragments labelling were performed as already reported (Lemoli *et al*, 1994).

RT-PCR and Southern blotting. The RT-PCR reactions were carried out using a modification of a previously described method (Erlich *et al*, 1991; Ferrari *et al*, 1993). Briefly, 1 μg of total RNA extracted from each sample was reverse transcribed using 400 U of M-MLV Reverse Transcriptase (GIBCO, BRL, Gaithesburg, Md.) and 1 μg of OligodT 15 primer (Boehringer, Mannheim) for 1 h at 42°C in 1 × RT buffer in a total volume of 30 μl. The cDNA was then heated at 95°C for 3 min and stored at 4°C. 1 μl of cDNA was subsequently amplified, adding 2·5 U of Taq polymerase (Promega), 0·5 μg of specific DR and RP in a total volume of 50 μl, in 10 mM Tris pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$, 200 μM dNTPs. The amplification was carried out performing 50 cycles using the follow conditions: denaturation at 45 s starting from the second cycle; annealing at 70°C for 2 min; extension at 72°C for 3 min. 10 μl of each sample was then electrophoresed on a 2% agarose gel. The gel was then denaturated in 0·2 N NaOH, 0·4 M NaCl for 45 min, neutralized in 25 mM phosphate buffer pH 6·5 for 45 min and then transferred by electroblotting on a positively charged nylon membrane. Hybridization of the blots with oligonucleotide-labelled probes was then performed. For each experiment the same amount of cDNA was amplified. To check the amount of cDNA in each sample, the β2 microglobulin gene was amplified by PCR with 35 cycles. A sample without RNA template was used as negative control to exclude amplified cDNA contamination. In each experiment the gel after ethidium bromide staining and the corresponding autoradiogram after hybridization were examined. The specificity of the amplification was confirmed by hybridization with an oligonucleotide probe.

Statistical analysis. The results are expressed as the mean ± the mean of the standard error (SEM) of at least three separate experiments. Statistical analysis was performed by mean of the paired nonparametric Wilcoxon rank sum test.

RESULTS

IL-11 mRNA expression in myeloid leukaemic cells

The constitutive expression of IL-11 mRNA in myeloid leukaemic cells was assessed by RT-PCR strategy (Figs 1 and 2). RNA extraction was performed from the MNC fraction in the absence of any further manipulation. Two AML samples showing < 90% blasts were purposely excluded from the

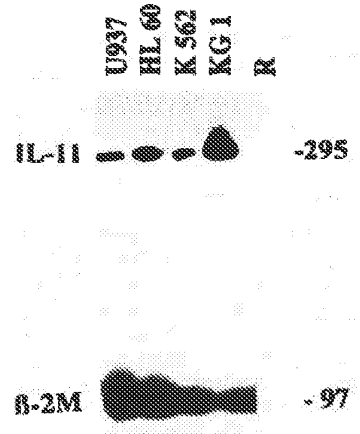


Fig 1. IL-11 mRNA expression obtained by RT-PCR in four myeloid cell lines. IL-11 mRNA was detected at the expected site (295 bp). A sample without RNA template, to exclude amplified cDNA contamination, served as negative control (R).

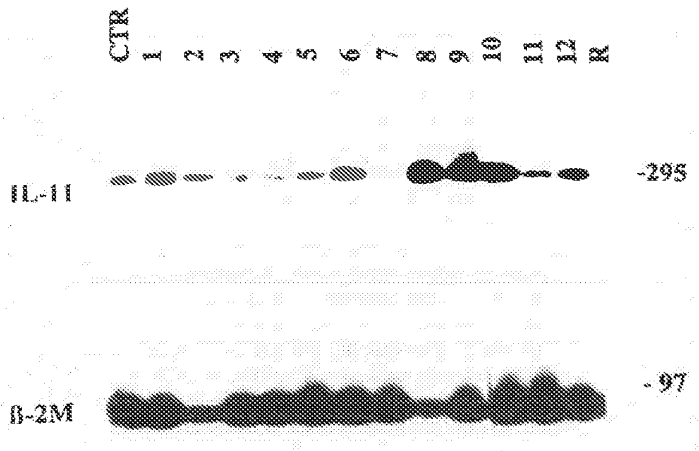


Fig 2. Constitutive expression of IL-11 mRNA assessed by RT-PCR in 12 AML samples. The two specimens containing < 90% of blast cells (see Table I) were excluded from analysis. A sample without RNA template, to exclude amplified cDNA contamination, was used as negative control while HL60 cells served as positive control.

study (nos. 3 and 10), whereas in cases 15 and 16 the genetic material for RNA analysis was not available.

Using specific primers for IL-11, we detected IL-11 transcript at the expected level in all the cell lines (Fig 1) and 11/12 AML primary samples (Fig 2).

To evaluate the possibility that the RT-PCR autoradiographic signal may be produced by rare contaminating peripheral blood mononuclear cells (PBMNC), several dilutions of PBMNC cDNA, corresponding to a possible sample contamination ranging from 1% to 100%, were also amplified using standard methods. Under these experimental conditions a sample contamination up to 50% did not produce a RT-PCR autoradiographic signal, even after very long exposure times of the autoradiograms (data not shown).

Effects of IL-11 on proliferation of leukaemic cell lines

The activity of 10 U/ml of IL-11 on the proliferation of three myeloid cell lines is shown in Fig 3. IL-11 did not induce the growth of KG1, K562 and HL60 cells in serum-containing (A) and serum-free (B) conditions. Concentrations of IL-11 up to 100 U/ml did not stimulate the cell lines as well, whereas higher concentrations were slightly inhibitory (data not shown). Because the consistent detection of IL-11 mRNA was suggestive of an IL-11-mediated autocrine growth loop, we then evaluated the spontaneous proliferation of leukaemic cells in presence of an anti-IL-11 Moab. Our results did not show any effect of the antisera, although the KG1 cell line was somewhat inhibited in the presence of exogenous FCS ($P = 0{\cdot}07$). In contrast to the lack of activity on its own, the addition of IL-11 to GM-CSF-, IL-3- or SCF-containing cultures, in serum-free medium, resulted in a marked increase of cell lines colony formation. A representative example is given in Fig 4. Notably, the synergistic activity of SCF and IL-11 in combination was totally abrogated by anti-IL-11 Moab (Fig 4). These results suggest that IL-11 does stimulate myeloid leukaemic cells by enhancing the proliferative response to other CSF.

CFU-L growth in response to IL-11

The clonogenic response of primary leukaemic samples to IL-11 is reported in Table II. To minimize non-specific cell interactions, each sample was plated in order to have about

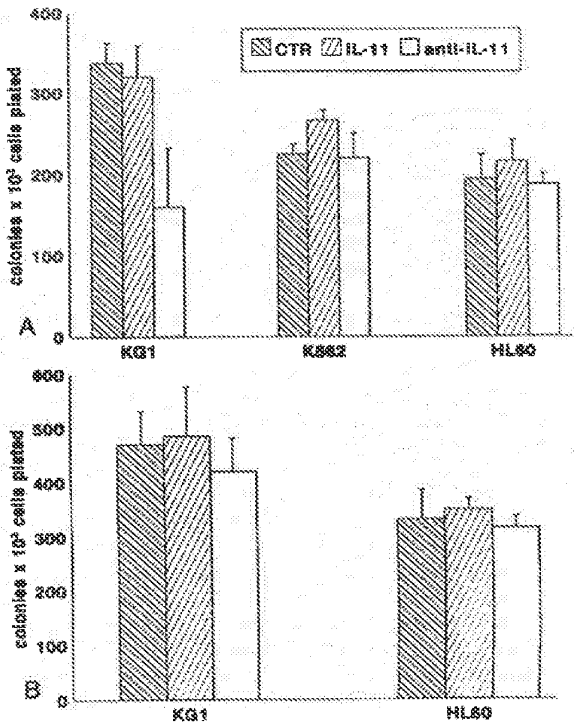


Fig 3. Lack of proliferative response of leukaemic cell lines to 10 U/ml of IL-11. A monoclonal antibody directed to IL-11 failed to inhibit the autonomous growth of cell lines. Cultures were performed in the presence (A) and the absence (B) of exogenous serum.

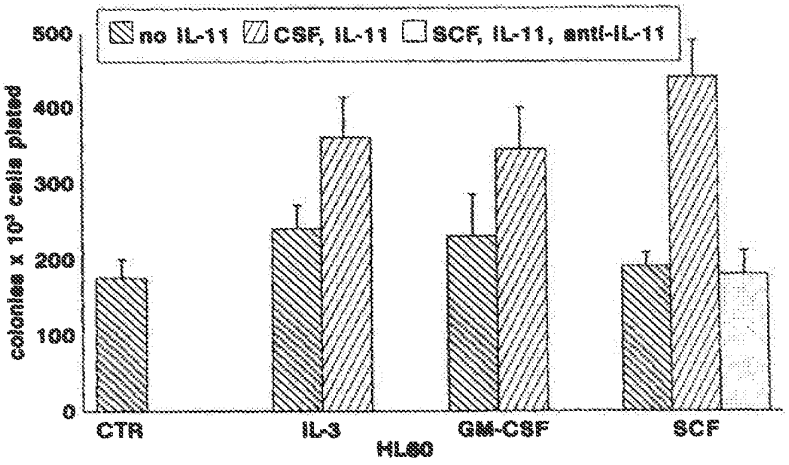


Fig 4. IL-11 enhances the IL-3, GM-CSF- and SCF-mediated clonogenic growth of HL60 cells in serum-free medium. The synergistic activity of SCF and IL-11 is abrogated by an anti-IL-11 MoAb.

100 colonies per dish. 6/8 AML patients showed some spontaneous growth, whereas the addition of either IL-11 (Table II) or anti-IL-11 antisera (data not shown) had little if any effect. Conversely, IL-11 enhanced in an additive or synergistic manner the proliferation of AML clonogenic cells in response to IL-3 in 8/8 cases (122 ± 37 colonies versus 60 ± 18; $P < 0{\cdot}05$) and GM-CSF in seven samples (138 ± 35 colonies versus 72 ± 21; $P < 0{\cdot}05$). More importantly, the combination of IL-11 and SCF resulted in a remarkable synergistic effect in seven cases (126 ± 43 colonies versus 24 ± 8 and 27 ± 12 colonies respectively, when SCF and IL-11 were used alone; $P < 0{\cdot}02$). The addition of the anti-IL-11 blocking MoAb to SCF and IL-11-supplemented cultures counteracted the stimulatory activity of the 2 CSFs (34 ± 8 colonies 126 ± 43, respectively). A three-factor combination (i.e. IL-11, SCF and IL-3) further enhanced CFU-L colony-forming ability which represented 79.5% of that induced by PHA-LCM (Table II). We did not find any effect on the size of CFU-L upon the addition of either IL-11 or SCF to other CSFs (data not shown).

IL-11 recruits AML cells in S-phase

Sixteen AML samples were assessed for their clonogenic growth in semisolid medium after cryopreservation. Eight samples generated a significant number of day 12–14 CFU-L (Table II) in response to PHA-LCM. Therefore the IL-11-mediated proliferation of the remaining eight AML cases was determined by the BRDU incorporation test (Table III). IL-11 alone did not augment the number of S-phase leukaemic cells as compared to control samples ($10{\cdot}5 \pm 2\%$ versus $10{\cdot}2 \pm 3\%$, respectively). Significant proliferation was induced by IL-3 and GM-CSF ($18 \pm 4\%$ and $16{\cdot}1 \pm 4\%$ BRDU positive cells, respectively; $P < 0{\cdot}05$) but not SCF. However, the addition of IL-11 to SCF significantly increased

Table II. Effect of recombinant human IL-11 on AML blast colony formation.

		No of colonies*									
Type	Patient	Medium (IMDM)	PHA-LCM	IL-11	SCF	IL-3	GM-CSF	IL-11, SCF	IL-11, IL-3	IL-11, GM-CSF	IL-11, SCF, IL-3
M1	4	2	65	4	20	27	40	80	64	130	110
M2	9	82	1405	98	70	170	180	338	314	308	930
M4	5	3	248	7	45	52	143	70	80	143	108
M4	13	31	95	64	25	45	80	288	250	246	252
M5a	10	11	145	15	16	90	64	132	119	158	327
M5a	11	0	514	7	0	24	16	22	40	30	62
M5a	12	25	48	14	10	64	35	16	80	49	212
M5b	14	0	100	11	8	6	17	59	26	38	84
Mean ± SEM†		19 ± 10	327 ± 163	27 ± 12	24 ± 8	60 ± 18	72 ± 21	126 ± 43	122 ± 37	138 ± 35	260 ± 101

*Results are expressed as the number of CFU-L $\times 10^5$ cells plated and represent the mean of triplicate counts.

† IL-3 andGM-CSF induced, as single cytokines, a significantly higher ($P < 0{\cdot}05$) CFU-L growth than control (medium alone) samples. The addition of IL-11 to other cytokines resulted in an increased blast colony formation as compared to the same CSF used alone (see Results).

Table III. Effect of recombinant human IL-11 on the number of leukaemic cells in S-phase.

		S-phase (%)*									
Type	Patient	Medium (IMDM)	PHA-LCM	IL-11	SCF	IL-3	GM-CSF	IL-11, SCF	IL-11, IL-3	IL-11, GM-CSF	IL-11, SCF, IL-3
M1	16	11	23	10	12	17	19	24	18	18	29
M1	8	10	18	17	10	11	16	20	19	18	19
M2	1	26	32	20	25	37	34	34	34	27	35
M2	3	2	ND	1·5	2·5	10	1·5	2·4	23	1·6	2·9
M4	2	2·8	ND	3·7	5	8	6	6·3	16·5	7·2	18·1
M4	6	17	12	18	14	21	25	30	27	26	39
M4	15	9	26	11	6	30	22	34	44	28	36
M5b	7	4	7	3	7	9	5	8	5	6	16
Mean ± SEM		10·2 ± 3	20 ± 4†	10·5 ± 2	10·2 ± 2	18 ± 4†	16·1 ± 4†	19·8 ± 4†	23·3 ± 4	16·5 ± 4	24·4 ± 4

* The number of cells in S-phase was determined by the BRDU assay, as described in Materials and Methods, after 3 d of liquid culture. BRDU positive cells at D0 were 3 ± 1%.

† Statistically significant compared to control samples or single cytokines (see Results).

the rate of DNA synthesis in four cases (> 20% increase compared to cultures supplemented with either SCF or IL-11 alone) (nos. 1, 6, 15 and 16). Moreover, IL-11 enhanced the response to leukaemic cells to IL-3 in three cases (nos. 3, 2 and 15).

DISCUSSION

IL-11 is a stimulatory protein for early lymphohaemopoietic stem/progenitor cells which has been shown to be a potent synergistic factor for intermediate-late-acting CSFs (Ogawa, 1993).

In contrast to normal haemopoietic cells, the role of IL-11 in the development of neoplastic cells is still poorly understood. When tested on myeloid leukaemia cells, IL-11 alone had little effect on several growth factor dependent cell lines as well as fresh samples from AML patients. However, IL-11 strongly enhanced the proliferative response of myeloid, but not lymphoid, leukaemia cells, to other CSFs and triggered AML cells in S-phase (Hu *et al*, 1993). Notably, IL-11 has been proposed recently as an autocrine growth factor for megakaryoblastic cell lines based upon the detection of IL-11 transcript and blocking experiments using anti-IL-11 MoAbs and antisense oligonucleotides (Kobayashi *et al*, 1993).

To address further the question of whether IL-11 plays a role in the development of AML, perhaps through an autocrine growth loop, we assessed the expression of IL-11 mRNA in a panel of fresh leukaemic samples and cell lines which do not belong to the megakaryoblastic differentiation lineage. Moreover, we evaluated the proliferative response of these cell populations to exogenous IL-11, alone and in various combinations with other cytokines such as the intermediate-late-acting CSF IL-3 and GM-CSF and the early-acting growth factor SCF which synergized with IL-11 in serum-free and serum-containing cultures on the proliferation of primitive $CD34^+$ $CD33^-$ DR^- normal marrow cells (Lemoli *et al*, 1993). The results presented here indicate that IL-11 is expressed consistently in continuously growing cell lines and myeloid leukaemia cells at diagnosis. To evaluate cytokine gene expression, RT-PCR technology was used. Because of the sensitivity of this assay, we tried to avoid the contamination of unwanted cells by including in the study 12 samples showing > 90% of leukaemic blasts in addition to cell lines. It should be also considered that IL-11 is expressed by stromal cells and limiting dilution experiments ruled out that IL-11 expression may be due to the potential contamination of PB mononuclear cells (see Results section). In this view, there is a large body of evidence that blast cells from AML patients express and produce a number of cytokines including GM-CSF (Young *et al*, 1987), interleukin-1 (Oster *et al*, 1989; Bradbury *et al*, 1990), interleukin-6 (Oster *et al*, 1989; Zhu *et al*, 1994) and SCF (Pietsch *et al*, 1992), which have been involved in the autonomous growth of the majority of AML samples. Among our study population, 14/16 AML samples did show a certain degree of spontaneous growth (colony formation = 6; S-phase = 8), which has been associated with autocrine CSF production.

However, similarly to normal target haemopoietic cells, IL-11 individually did not induce the proliferation of AML blast cells and blocking experiments in serum-free conditions did not provide evidence that autocrine production and secretion of IL-11 may contribute to the growth of leukaemic cells. The lack of correlation between constitutive expression of cytokine genes, their production and autocrine growth in AML cells has been previously addressed and it has been suggested that the presence of cytokine transcripts may be induced by *in vitro* manipulation of leukaemic cells (Kaufmann *et al*, 1988). For this reason, we did not attempt to remove rare contaminating cells and RNA extraction was performed from the MNC fraction in the absence of any further step. In the presence of IL-11 mRNA the adsorption of IL-11 by AML cells themselves or the activity of an intracytoplasmic loop may be hypothesized, although Kobayashi *et al* (1993) have shown the lack of inhibition

of AML cell lines growth by antisense oligonucleotides directed to IL-11. In fact, in previous studies M-CSF transcript has been found in a large number of AML specimens studied, whereas no M-CSF activity has been detected in culture supernatants (Young *et al.* 1988). Alternatively, it is possible that IL-11 mRNA is not translated or it is translated into an inactive protein.

In the present paper, both the clonogenic and the BRDU incorporation assays demonstrated that IL-11 enhanced the IL-3- and to a lesser extent, GM-CSF-mediated proliferation of AML progenitor cells. Most interestingly, IL-11 synergized with the early-acting growth factor SCF, and this combination was as effective as the other two-factor combinations tested in supporting the growth of AML cells, although the two cytokines showed no activity on their own. In this regard, the proliferative response of leukaemic cells to IL-11 appears to be similar to that of their normal counterparts, as judged by clonogenic assays and short-term liquid cultures of early $CD34^+$ and $CD34^+$ $CD33^-$ DR^- (Lemoli *et al.* 1993), and supports previous studies on myeloid leukaemia cells (Hu *et al.* 1993). Moreover, in most of our patients, the simultaneous presence in the culture of three growth factors (i.e. IL-11, SCF and IL-3) resulted in an increased colony number as compared with the single most active factor or two-factor combinations, and the magnitude of proliferation was comparable to that of PHA-LCM-stimulated cultures. Our results suggest that AML cells retain a responsiveness to the study CSFs which reflects the features of normal haemopoietic progenitors and confirm earlier investigations where the intermediate-late-acting growth factors IL-3, GM-CSF and G-CSF were tested (Lemoli *et al.* 1991). Thus, because of the lack of differential activity on normal and leukaemic cells, IL-11 may not be useful in bringing AML blasts into cell cycle prior to exposure to cycle-specific drugs (Hu *et al.* 1993).

In summary, although earlier papers have demonstrated that IL-11 is an autocrine growth factor for megakaryoblastic cells, the results presented here suggest that IL-11 may play a role in the development of AML cells by acting as a potent synergistic factor. Further studies are warranted to investigate whether IL-11 acts through an autocrine growth loop or in a paracrine fashion.

ACKNOWLEDGMENT

The research was supported by MURST 40% and MURST 60%.

REFERENCES

Baumann, H. & Schendel, P. (1991) Interleukin-11 regulates the hepatic expression of the same plasma protein genes as interleukin-6. *Journal of Biological Chemistry*, **266**, 20121–20127.

Bennett, J.M., Canovsky, D., Daniel, M.T., Flandrin, G., Galton, D.A.G., Gralnick, H.R. & Sultan, C. (1985) Proposed revised criteria for the classification of acute myeloid leukaemia. *Annals of Internal Medicine*, **103**, 620–625.

Bradbury, D., Bowen, G., Kozlowski, R., Reilly, I. & Russel, N. (1990) Endogenous interleukin-1 can regulate autonomous growth of acute myeloblastic leukaemia cells by inducing autocrine secretion of GM-CSF. *Leukemia*, **4**, 44–47.

Bruno, E., Briddel, R.A., Cooper, R.J. & Hoffmann, R. (1991) Effects of recombinant interleukin 11 on human megakaryocyte progenitor cells. *Experimental Hematology*, **19**, 378–381.

Du XX, Keller, D.C., Maze, R. & Williams, D.A. (1993a) Comparative effects of in vivo treatment using interleukin-11 and stem cell factor on reconstitution in mice following bone marrow transplantation. *Blood*, **82**, 1016–1022.

Du, XX, Neben, T., Goldman, S & Williams, D.A. (1993b) Effects of recombinant human interleukin-11 on hematopoietic reconstitution in transplanted mice: acceleration of recovery of peripheral blood neutrophils and platelets. *Blood*, **81**, 27–34.

Du, XX, & Williams, D.A. (1994) Interleukin-11: a multifunctional growth factor derived from the hematopoietic microenvironment. *Blood*, **83**, 2023–2030.

Erlich, H.A., Gelfand, D. & Sninsky, J.J. (1991) Recent advances in the polymerase chain reaction. *Science*, **252**, 1643–1650.

Ferrari, S., Grande, A., Manfredini, R., Taglafico, E., Zucchini, P., Torelli, G. & Torelli, U. (1993) Expression of interleukin 1, 3, 6, stem cell factor and their receptors in acute leukemia blast cells and in normal peripheral lymphocytes and monocytes. *European Journal of Hematology*, **50**, 141–148.

Hu, J.P., Cesano, A., Santoli, D., Clark, S.C. & Hoang, T. (1993) Effects of interleukin-11 on the proliferation and cell cycle status of myeloid leukemic cells. *Blood*, **81**, 1586–1592.

Kaufmann, D.C., Baer, M., Zhi, G., Wang, Z. & Preisler, H.D. (1988) Enhanced expression of the granulocyte-macrophage colony-stimulating factor gene in acute myelocytic leukemia following in vitro blast cell enrichment. *Blood*, **72**, 1329–1332.

Keller, D.C., Du, X.X., Srour, E.F., Hoffmann, R. & Williams, D.A. (1993) Interleukin-11 inhibits adipogenesis and stimulates myelopoiesis in long-term marrow cultures. *Blood*, **82**, 1428–1435.

Kobayashi, S., Teramura, M., Sugawara, I., Oshimi, K. & Mizoguchi, H. (1993) Interleukin-11 acts as an autocrine growth factor for human megakaryoblastic cell lines. *Blood*, **81**, 889–893.

Leary, A.G., Zeng, H.Q., Clark, S.C. & Ogawa, M. (1992) Growth factor requirements for survival in G0 and entry into the cell cycle of primitive human hemopoietic progenitors. *Proceedings of the National Academy of Sciences of the United States of America*, **89**, 4013–4017.

Lemoli, R.M., Fogli, M., Fortuna, A., Motta, M.R., Rizzi, S., Benini, C. & Tura, S. (1993) Interleukin-11 stimulates the proliferation of human hematopoietic $CD34^+$ and $CD34^+CD^-DR^-$ cells and synergizes with stem cell factor, interleukin-3, and granulocyte-macrophage colony-stimulating factor. *Experimental Hematology*, **21**, 1668–1672.

Lemoli, R.M., Fortuna, A., Grande, A., Gamberi, B., Bonsi, L., Fogli, M., Amabile, M., Cavo, M., Ferrari, S. & Tura, S. (1994) Expression and functional role of c-kit ligand (SCF) in human multiple myeloma (MM) cells. *British Journal of Haematology*, **88**, 760–769.

Lemoli, R.M., Gulati, S.C., Strife, A., Lambek, C., Perez, A. & Clarkson, B.D. (1991) Proliferative response of acute myeloid leukemia cells and normal marrow enriched progenitor cells to human recombinant growth factors IL-3, GM-CSF and G-CSF alone and in combination. *Leukemia*, **5**, 386–391.

McDonald, R.J., Swift, J.H., Przybyla, A.E. & Chirgwin, J.M. (1987) Isolation of RNA using guanidinium salts. *Methods in Enzymology*, **152**, 219–221.

Musashi, M., Yang, Y.C., Paul, S.R., Clark, S.C., Sudo, T. & Ogawa, M. (1991) Direct and synergistic effects of interleukin 11 on murine hemopoiesis in culture. *Proceedings of the National Academy of Sciences of the United States of America*, **88**, 765–769.

Ogawa, M. (1993) Differentiation and proliferation of hematopoietic stem cells. *Blood*, **81**, 2844–2853.

Oster, W., Cicco, N.A., Klein, H., Hirano, T., Kishimoto, T., Lindemann, A., Mertelsmann, R.H. & Herrmann, F. (1989) Participation of cytokines, interleukin-6, tumor necrosis factor-α and interleukin 1β secreted by acute myelogenous leukemia blasts in autocrine and paracrine leukemia growth control. *Journal of Clinical Investigation*, **84**, 451–457.

Paul, S.R., Bennet, F., Calvetti, J.A., Kelleher, K., Wood, C.R., O'Hara, R.M., Leary, A.C., Sibley, B., Clark, S.C., Williams, D.A. & Yang, Y.C. (1990) Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymphopoietic and hematopoietic cytokine. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 7512–7516.

Pietsch, T., Kyas, U., Steffens, U., Yakisan, E., Hadam, M.R., Ludwig, W.D., Zsebo, K. & Welte, K. (1992) Effects of human stem cell factor (c-kit ligand) on proliferation of myeloid leukemia cells: heterogeneity in response and synergy with other hematopoietic growth factors. *Blood*, **80**, 1199–1206.

Yonemura, Y., Kawakita, M., Masuda, T., Fujimoto, K., Kato, K. & Takasuki, K. (1992) Synergistic effects of interleukin 3 and interleukin 11 on murine megakaryopoiesis in serum free culture. *Experimental Hematology*, **20**, 1011–1016.

Young, D.C., Demetri, G.D., Ernst, T.J., Cannistra, S.A. & Griffin, J.D. (1988) In vitro expression of colony-stimulating factor genes by human acute myeloblastic leukemia. *Experimental Hematology*, **16**, 378–382.

Young, D.C., Wagner, K. & Griffin, J.D. (1987) Constitutive expression of the granulocyte-macrophage colony-stimulating factor gene in acute myeloblastic leukemia. *Journal of Clinical Investigation*, **79**, 100–106.

Zhu, M.Y., Bradbury, D.A., Keith, F.J. & Russel, N. (1994) Absence of retinoblastoma protein expression results in autocrine production of interleukin-6 and promotes the autonomous growth of acute myeloid leukemia blast cells. *Leukemia*, **8**, 1982–1988.

Recombinant Human Anti-human IL11 Antibody

Cat. No: MHH-551

PRODUCT INFORMATION

Product Overview	*Recombinant Human Antibody reacts with an antigen Human IL11, expressed in HEK 293 cells. Expressed as the combination of a heavy chain (HC) containing VH from anti-IL11 mAb and CH1-3 region of human IgG1 and a light chain (LC) encoding VL from anti-IL11 mAb and CL of human kappa light chain. Exists as a disulfide linked dimer of the HC and LC hetero-dimer under non-reducing condition.*
Host	*Human*
Specificity	*Tested positive against native Human interleukin 11*
Target	*IL11*
Immunogen	*Human interleukin 11*
Antibody Isotype	*IgG1*
Derivation	*Human*
Species Reactivity	*Human*
Type	*IgG*
Expression Host	*HEK 293*
Purity	*>95% by HPLC*
Applications	*Products can be used for: Flow Cytometry; Neutralization; Functional Study*
Formulation	*Functional Grade Purified (low endo, azide-free)*
Storage	*Store at 4°C for up to 3 months. For longer term storage aliquot into small volumes and store at -20°C.*

ANTIGEN INFORMATION

Introduction	*The protein encoded by this gene is a member of the gp130 family of cytokines. These cytokines drive the assembly of multisubunit receptor complexes, all of which contain at least one molecule of the transmembrane signaling receptor IL6ST (gp130). This cytokine is shown to stimulate the T-cell-dependent development of immunoglobulin-producing B cells. It is also found to support the proliferation of hematopoietic stem cells and megakaryocyte progenitor cells. Alternatively spliced transcript variants encoding distinct isoforms have been found for this gene.*
Alternative Names	*IL11; interleukin 11; AGIF; IL-11; interleukin-11; oprelvekin; adipogenesis inhibitory factor*
Gene ID	*3589*
UniProt ID	*A8K3F7*

RELATED PRODUCTS

Cat #	Description
NEUT-1145CQ	Recombinant Mouse Anti-IL11 Antibody (CBL088)
NEUT-1150CQ	Recombinant Rat Anti-Il11 Antibody (CBL582)
NEUT-1149CQ	Recombinant Rat Anti-Il11 Antibody (CBL089)
MHH-551-F(E)	Recombinant Human Anti-human IL11 Antibody Fab Fragment
TP-033CL	Oprelvekin
NEUT-1142CQ	Recombinant Mouse Anti-IL11 Antibody (MM0361-9T27)
NEUT-1146CQ	Recombinant Mouse Anti-IL11 Antibody (CBL265)
MOB-0777CT	Recombinant Mouse anti-Human IL11 Monoclonal antibody (LU73)

Cancer Cell
Article

Interleukin-11 Is the Dominant IL-6 Family Cytokine during Gastrointestinal Tumorigenesis and Can Be Targeted Therapeutically

Tracy L. Putoczki,[1,6,10,11,*] Stefan Thiem,[1,10,11] Andrea Loving,[1] Rita A. Busuttil,[3,4,5] Nicholas J. Wilson,[2] Paul K. Ziegler,[7] Paul M. Nguyen,[1,10,11] Adele Preaudet,[1,10,11] Ryan Farid,[1,10,11] Kirsten M. Edwards,[2] Yeliz Boglev,[1] Rodney B. Luwor,[6] Andrew Jarnicki,[1,12] David Horst,[8] Alex Boussioutas,[3,4,5] Joan K. Heath,[1,10,11] Oliver M. Sieber,[1,10,11] Irina Pleines,[9] Benjamin T. Kile,[9] Andrew Nash,[2] Florian R. Greten,[7] Brent S. McKenzie,[2] and Matthias Ernst[1,6,10,11,*]
[1]Ludwig Institute for Cancer Research, Melbourne, VIC 3050, Australia
[2]CSL, Ltd., Melbourne, VIC 3050, Australia
[3]Cancer Genetics and Genomics Laboratory, Peter MacCallum Cancer Centre, East Melbourne, VIC 3002, Australia
[4]The Sir Peter MacCallum Department of Oncology, Peter MacCallum Cancer Centre, The University of Melbourne, Melbourne, VIC 3010, Australia
[5]Department of Medicine
[6]Department of Surgery
Royal Melbourne Hospital, The University of Melbourne, VIC 3050, Australia
[7]Institute of Molecular Immunology, Technical University Munich, Munich 81675, Germany
[8]Institute of Pathology, Ludwig-Maximilian University, Munich 80337, Germany
[9]Walter and Eliza Hall Institute of Medical Research, VIC 3052, Australia
[10]Present address: Department of Medical Biology, The University of Melbourne, VIC 3050, Australia
[11]Present address: Walter and Eliza Hall Institute of Medical Research, VIC 3052, Australia
[12]Present address: School of Biomedical Sciences and Pharmacy, University of Newcastle, NSW 2308, Australia
*Correspondence: tracy.putoczki@wehi.edu.au (T.L.P.), matthias.ernst@wehi.edu.au (M.E.)
http://dx.doi.org/10.1016/j.ccr.2013.06.017

SUMMARY

Among the cytokines linked to inflammation-associated cancer, interleukin (IL)-6 drives many of the cancer "hallmarks" through downstream activation of the gp130/STAT3 signaling pathway. However, we show that the related cytokine IL-11 has a stronger correlation with elevated STAT3 activation in human gastrointestinal cancers. Using genetic mouse models, we reveal that IL-11 has a more prominent role compared to IL-6 during the progression of sporadic and inflammation-associated colon and gastric cancers. Accordingly, in these models and in human tumor cell line xenograft models, pharmacologic inhibition of IL-11 signaling alleviated STAT3 activation, suppressed tumor cell proliferation, and reduced the invasive capacity and growth of tumors. Our results identify IL-11 signaling as a potential therapeutic target for the treatment of gastrointestinal cancers.

INTRODUCTION

The onset and progression of cancer is facilitated by complex interactions between neoplastic cells and the heterogeneous stromal cell populations that are present in the tumor microenvironment (Egeblad et al., 2010; Hanahan and Coussens, 2012). While this is best documented for gastrointestinal (GI) cancers associated with persistent inflammation, the tumor microenvironment can also promote the growth of sporadic cancers arising from tumor intrinsic oncogenic mutations (Grivennikov et al., 2012). Studies utilizing knockout mice have begun to unravel the complex interplay between the neoplastic and stromal cells and have highlighted pivotal roles for inflammatory cytokines. In turn, these cytokines collectively promote cancer hallmark capabilities,

Significance

STAT3 activation is linked to poor survival in patients with cancer and is thought to arise primarily from elevated IL-6. Accordingly, inhibitors of IL-6 signaling are in clinical trials for a number of epithelial cancers. Using mouse models of inflammation-associated and sporadic gastrointestinal cancers, we have discovered that the IL-11/STAT3 signaling axis is a more potent driver of tumor progression than IL-6. Pharmacologic inhibition of IL-11/STAT3 in mouse models of gastrointestinal cancer and human tumor cell line xenografts inhibited the invasive capacity of neoplastic cells and reduced tumor growth. Importantly, IL-11 inhibition had no impact on hematopoiesis, an undesirable side effect of systemic STAT3 inhibition. Our data provide support for the clinical development of IL-11 signaling antagonists for the treatment of epithelial cancers.

including proliferation, angiogenesis, and metastasis, while simultaneously inducing resistance of neoplastic cells to death stimuli and immune destruction (Hanahan and Weinberg, 2011).

The interleukin (IL)-6 family of cytokines is defined by the shared use of the gp130 receptor β-subunit. Included within this family are IL-6, recognized for its role as a systemic acute phase mediator (Heinrich et al., 1990), and IL-11, which promotes platelet production (Teramura et al., 1992). More recently, both of these cytokines have been linked to the development of epithelial cancers (Matsuo et al., 2003; Nakayama et al., 2007). While activated myeloid cells are thought to produce most of the IL-6 in the tumor microenvironment, autocrine IL-6 signaling in neoplastic epithelial cells has also recently been documented (Gao et al., 2007; Grivennikov et al., 2009). IL-11 on the other hand is produced by cancer-associated fibroblasts (CAFs) in patients with colorectal cancer (CRC) and by myeloid cells and can be upregulated in cancer cells as part of an autocrine signaling loop (Calon et al., 2012; Ernst et al., 2008; Schwitalla et al., 2013; Shin et al., 2012). Although the engagement of the gp130 receptor by either IL-6 or IL-11 induces transient activation of Janus kinases (JAK) and the latent transcription factor STAT3, tissue responses are dependent on either the presence of soluble IL-6 receptor (R) or expression of the membrane-associated IL-6Rα and IL-11Rα receptors (Becker et al., 2004; Heinrich et al., 1998).

Excessive STAT3 activation is a feature of the majority of solid cancers and is frequently associated with elevated cytokine expression, including IL-6 and IL-11 (Ernst et al., 2008; Grivennikov et al., 2009). However, some epithelial malignancies are also associated with activating somatic mutations in the genes encoding STAT3, gp130, and associated JAK1/2, as well as epigenetic silencing of the *SOCS3* gene, which encodes a critical negative regulator for gp130 cytokine signaling (Casanova et al., 2012; He et al., 2003; Rebouissou et al., 2009). In GI cancers, excessive STAT3 activation is also linked to tumor invasion and nodal metastasis and predicts poor patient survival (Deng et al., 2010; Kim et al., 2009; Morikawa et al., 2011). We have previously shown that epithelial STAT3 activation in mice promotes inflammation-associated gastric cancer (GC) and colitis-associated colorectal cancer (CAC) (Bollrath et al., 2009; Ernst et al., 2008). Furthermore, genetic reduction of STAT3 expression diminished tumor burden in these models, and suggested that pharmacologic targeting of the gp130/STAT3 signaling pathway may confer significant therapeutic benefits in the treatment of these malignancies.

The concept of combating tumor progression through pharmacologic inhibition of growth-promoting cytokines is emerging as a therapeutic opportunity that bypasses the difficulties of targeting intracellular signaling molecules and transcription factors. To date, much focus has been placed on antagonizing the activity of IL-6, with clinical trials for ovarian, renal, prostate, and breast cancers underway (Guo et al., 2012; Puchalski et al., 2010). Here, we investigate the role of the related cytokine IL-11 in GI tumorigenesis.

RESULTS

Increased IL-11 Expression in Human GI Cancers Is Associated with Excessive STAT3 Activation

Although elevated IL-6 expression is linked to excessive STAT3 activation associated with poor survival in patients with CRC, less is known about the role of IL-11 in these cancers (Esfandi et al., 2006; Morikawa et al., 2011). We therefore compared the expression of *IL6* and *IL11* in a panel of 14 primary CRC samples (Figure 1A) and in 16 primary human GC samples (Figure S1A available online). We found that both cytokines were consistently elevated in tumor tissue compared to unaffected GI tissues from the same patients. To examine the relationship between epithelial STAT3 activation and the expression of inflammatory cytokines associated with tumorigenesis, we assessed pTyr-STAT3 staining as a marker of activated STAT3, in additional CRC samples, and found that only *IL11* mRNA expression significantly correlated with high epithelial STAT3 activation (Figures 1B and S1B). Within this high STAT3 patient cohort, we found no correlation between *IL6* and *IL11* expression (not shown). Because we consistently observed heterogeneous STAT3 activation within tumor samples, we characterized the location of pTyr-STAT3 positive cells within a panel of 59 resected primary CRC samples. We observed strong pTyr-STAT3 staining in the tumor core in 39% (23 of 59) of samples. When we scored invasive regions of these tumors separately, we found strong pTyr-STAT3 staining in 61% (36 of the 59; Figure 1C) of samples, suggesting a potent trigger for STAT3 activation near the invasive front. Similarly, in a panel of GC samples, we observed high levels of epithelial STAT3 activation in 26% (33 of 125) of the specimens (Figure S1C). Collectively, these observations suggest that IL-11-mediated STAT3 activation may be associated with epithelial tumorigenesis and invasion of neoplastic cells into the submucosa.

IL-11 Signaling Is a Dominant Driver of Inflammation-Associated Colon Cancer in Mice

To compare the role of IL-6 and IL-11 during CRC, we induced CAC in mice. In the CAC model (Figure 2A), a single injection of the alkylating mutagen azoxymethane (AOM) leads to sporadic induction of a number of mutations, including mis-sense mutations in *Ctnnb1* in intestinal epithelial cells, resulting in stabilization of the corresponding β-catenin protein and aberrant activation of the Wnt-signaling pathway (Neufert et al., 2007; Tanaka et al., 2005). Subsequent repetitive administration of the luminal irritant dextran sulfate sodium (DSS) promotes "flares" of inflammation, akin to those observed in chronic ulcerative colitis, which augment colonic tumor development in mice (Wirtz et al., 2007). Similar to our observation in human CRC, we detected increased IL-11 and pTyr-STAT3 protein levels in the distal colonic tumors of wild-type (WT) mice on day 72 of the CAC protocol (Figure 2B). Because the progression of CAC-induced colonic neoplasias is mediated by STAT3 (Bollrath et al., 2009; Grivennikov et al., 2009), we compared the requirement for the STAT3 activating cytokines IL-6 and IL-11 during this process. Using serial endoscopy, we detected macroscopic tumors in WT and $Il6^{KO}$ mice by day 47, whereas tumor onset was substantially delayed in $Il11ra1^{KO}$ and $Il6^{KO}$;$Il11ra1^{KO}$ compound mutant mice (Figure 2C). Although the absence of IL-6 signaling resulted in a lower frequency of colonic adenomas in $Il6^{KO}$ mice compared to WT mice (Grivennikov et al., 2009), tumors were almost completely absent in the colons of $Il11ra1^{KO}$ mice at autopsy, irrespective of the presence of IL-6 (Figures 2C–2E). Consistent with a dominant role for IL-11-mediated STAT3 activation in tumor formation we observed a reduction

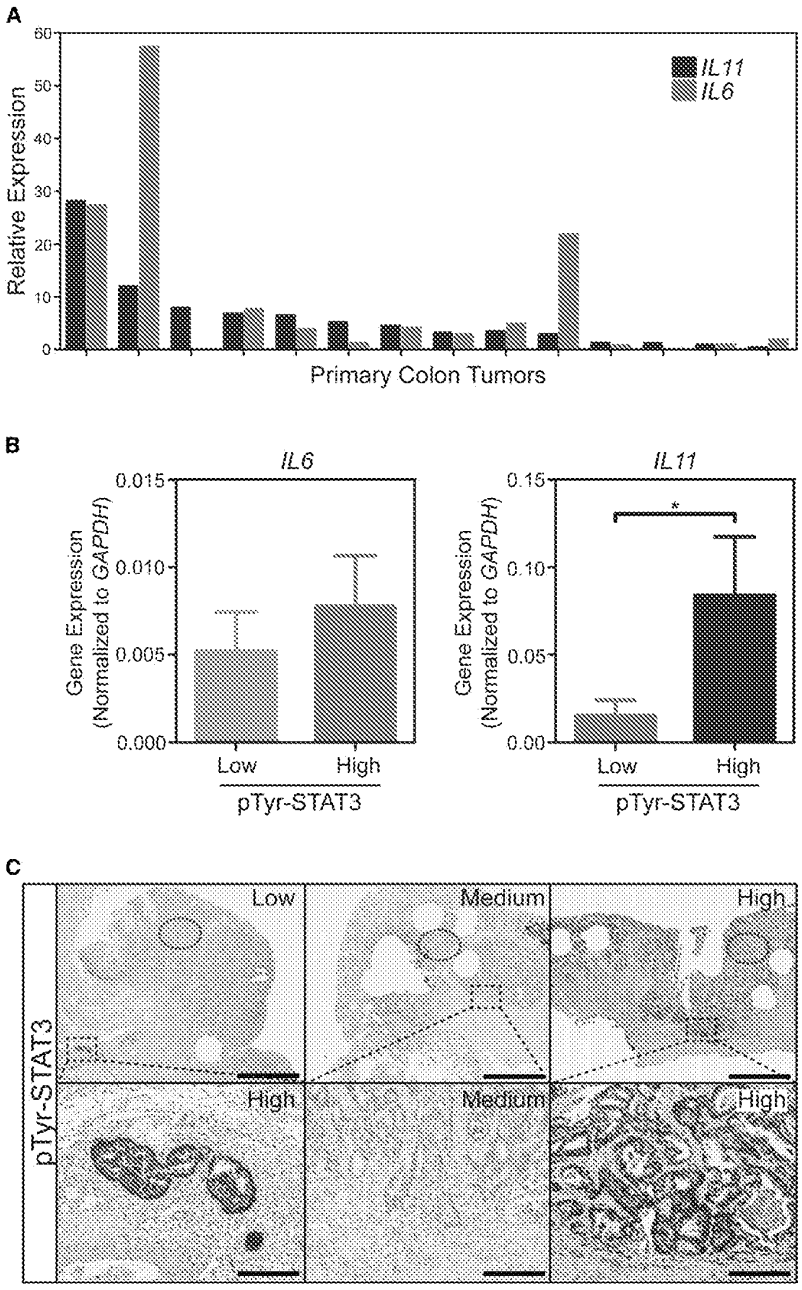


Figure 1. Elevated *IL11* Expression in Human CRC Is Associated with Excessive STAT3 Activation

(A) *IL6* and *IL11* mRNA expression in 14 individual human CRC samples. Data were normalized to *GAPDH* expression and are shown as fold change compared to unaffected colon tissue from the same patients.

(B) STAT3 activation in 46 human CRC samples was determined by immunohistochemistry for pTyr-STAT3 to classify samples with low (24 samples) or high (22 samples) STAT3 activation followed by analysis for *IL6* and *IL11* mRNA expression. Data were normalized to *GAPDH* expression and are shown as mean ± SEM (*$p < 0.05$).

(C) Representative pTyr-STAT3 immunohistochemistry depicting STAT3 activation in the core region (oval) or invasive front (rectangle) of representative human CRC samples. The bottom row depicts higher magnifications of the boxed areas. Scale bars: 3 mm (top row); 300 μm (bottom row). See also Figure S1.

in pTyr-STAT3 levels within isolated colonic epithelial cells of *Il11ra1*^{KO} mice following a single injection of AOM and one cycle of DSS (Figure S2A). Similarly, distal colonic tumors from *Il11ra1*^{KO} and *Il6*^{KO};*Il11ra1*^{KO} mice showed reduced levels of pTyr-STAT3 and reduced expression of the downstream target protein BCL-2, compared to WT and *Il6*^{KO} mice (Figure 2F).

To further validate the requirement for IL-11 signaling in the CAC model, we utilized homozygous *gp130*$^{F/F}$ mutant mice that expressed gp130(Y757F) (Tebbutt et al., 2002). gp130(Y757F) cannot interact with gp130's negative regulator SOCS3, leading to enhanced IL-6 family cytokine-mediated STAT3 activation (Figure S2B). First, we confirmed that IL-11 expression was elevated in the CAC tumors of *gp130*$^{F/F}$ mice (Figure S2C). We next inhibited IL-11 or IL-6 signaling genetically in *gp130*$^{F/F}$ mice and revealed a dominant role for IL-11 driven STAT3 activation in promoting CAC-induced tumorigenesis (Figures S2D–S2G). Moreover, the reduction in tumor burden observed in *Il11ra1*^{KO} and *gp130*$^{F/F}$;*Il11ra1*^{KO} mice coincided with a reduction in the submucosal inflammation compared to tumor-bearing WT and *gp130*$^{F/F}$ mice (Figures 2E and S2F). Collectively, these observations establish IL-11 signaling as a driver of inflammation-associated tumorigenesis that is more profound than that previously ascribed to IL-6.

Colonic Tumorigenesis Is Not Dependent on IL-11-Responsive Hematopoietic Cells

Physical disruption of the mucosal barrier by DSS exposes innate immune cells of the submucosa to luminal antigens and provides a stimulus for the production of pro-inflammatory cytokines that can fuel epithelial tumor progression (Hanahan and Coussens, 2012). To determine whether the tumor-promoting function of IL-11 was mediated by cells of hematopoietic origin, we generated reciprocal bone marrow chimeras. Following complete engraftment, recipient mice were subjected to only the first two DSS cycles of the CAC protocol (Figure 2A) and were then monitored by serial endoscopy. We found that tumor burden in lethally

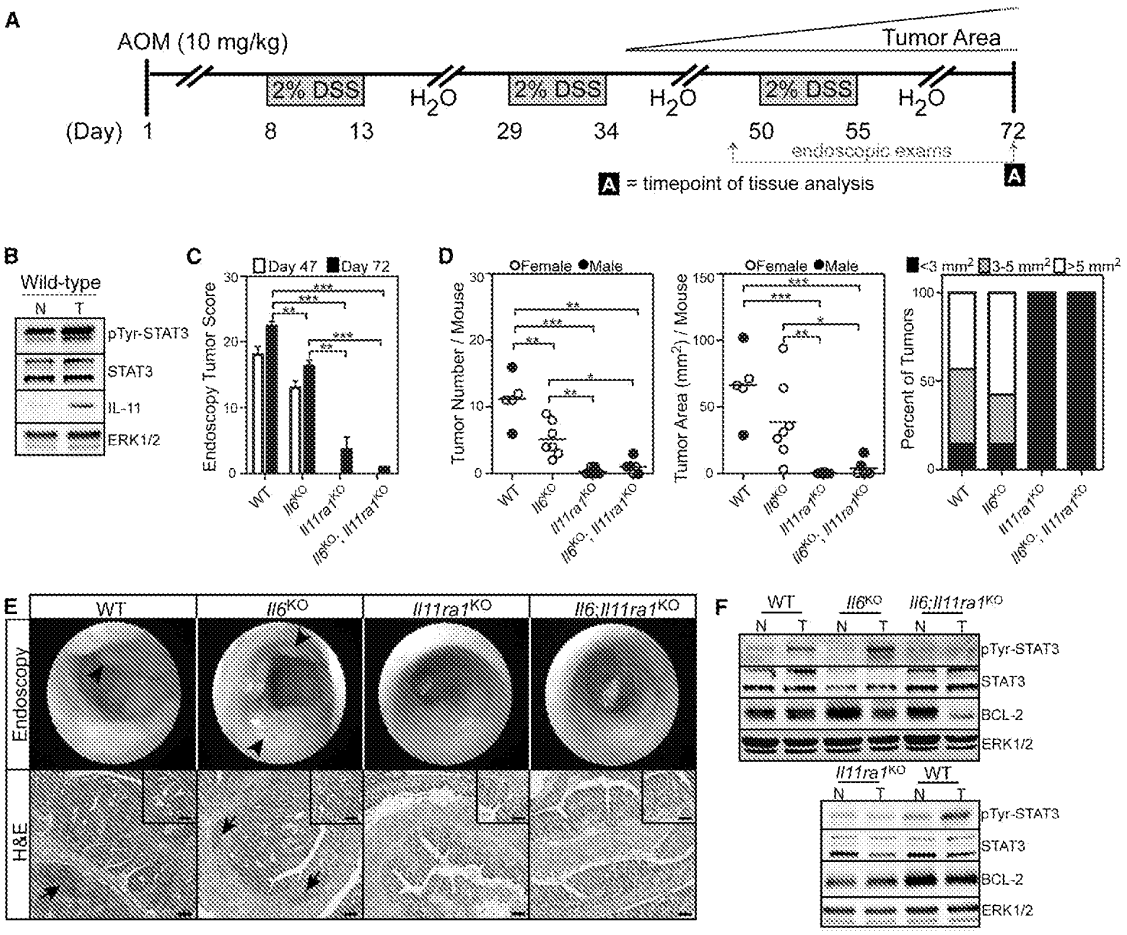


Figure 2. IL-11 Is the Dominant IL-6 Family Cytokine during CAC in Mice

(A) Schematic representation of the CAC model. A single injection of AOM is followed by repeated administration of DSS provided ad libitum in the drinking water. The formation of distal colonic tumors is then monitored by endoscopy at the indicated time points.

(B) Representative immunoblot analysis of distal colonic tumor (T) and adjacent nontumor (N) tissue from WT mice collected on day 72 of the CAC model. ERK1/2 was used as a loading control.

(C) Tumor burden of mice of the indicated genotypes was scored by endoscopy at day 47 and 72 of the CAC model; data are presented as mean ± SEM ($n \geq 4$ mice per cohort; $^{**}p < 0.01$, $^{***}p < 0.001$).

(D) Colonic tumor burden from individual mice of the indicated genotypes at autopsy on day 72. Horizontal lines refer to mean values ($n \geq 5$ mice per cohort; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

(E) Representative endoscopy images (top row) of distal colonic tumors (arrowhead) on day 72 of the CAC model. Representative images of corresponding hematoxylin and eosin stained (H&E) sections (bottom row) with regions of inflammatory cell infiltration (arrows) in the tumor microenvironment. Scale bars: 200 μm; inset: 130 μm.

(F) Representative immunoblot analysis of colonic tumors (T) and adjacent nontumor (N) tissues for pTyr-STAT3 and BCL-2 from mice of the indicated genotypes collected on day 72 of the CAC model. ERK1/2 was used as a loading control.

See also Figure S2.

irradiated WT recipients reconstituted with *Il11ra1*^{KO} bone marrow was comparable to that of their WT littermates reconstituted with WT bone marrow (Figures 3A and 3B). In contrast, loss of IL-11 signaling in the nonhematopoietic cells of recipient *Il11ra1*^{KO} hosts rendered mice resistant to CAC. To examine whether loss of IL-11 signaling altered the recruitment of immune cells into the tumor microenvironment, we stained colons for CD45-positive hematopoietic cells, F4/80-positive macrophages (Figures 3C and 3D), Gr1-positive neutrophils, and CD3-positive lymphocytes (Figure S3A). This revealed that tumor-associated submucosal immune cell infiltrates occurred irrespective of the capacity of the hematopoietic cells to respond to IL-11. Likewise, we observed that the expression of many inflammatory cytokines in tumors and unaffected colonic mucosa were not affected by the absence of IL-11-responsive hematopoietic cells (Figure S3B). In contrast, *Il11ra1* ablation in the nonhematopoietic compartments of the recipient hosts led to a reduction in submucosal inflammation

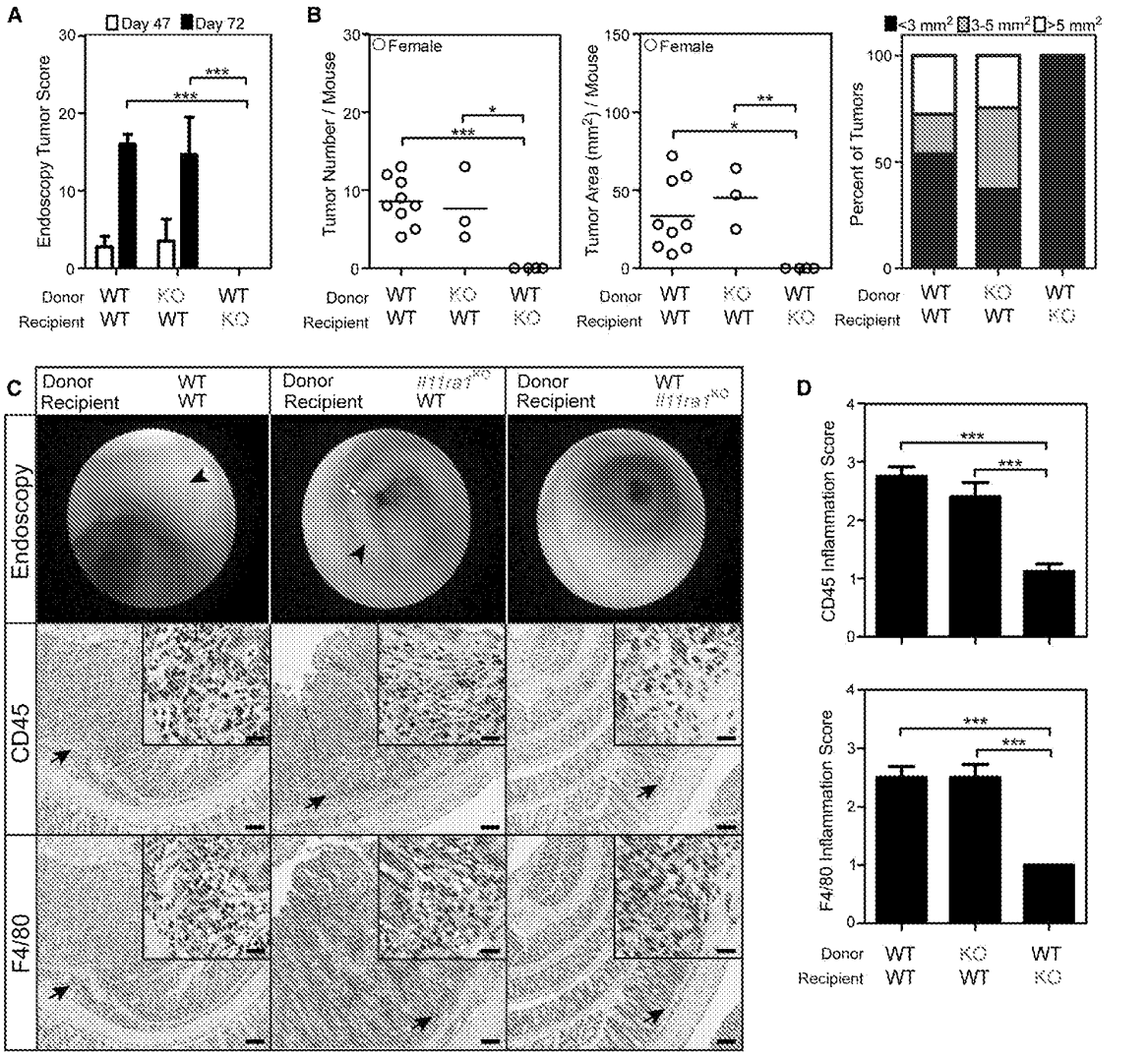


Figure 3. IL-11-Responsive Hematopoietic Cells Are Dispensable for Tumorigenesis

(A) Tumor burden of reciprocal WT and *Il11ra1*^{KO} (KO) bone marrow chimeras was scored by endoscopy at day 47 and 72 of the CAC model and is depicted as mean ± SEM ($n \geq 4$ mice per cohort; ***$p < 0.001$).

(B) Colonic tumor burden in individual reciprocal WT and *Il11ra1*^{KO} (KO) bone marrow chimeras at autopsy on day 72 of the CAC model. Horizontal lines refer to mean values ($n \geq 3$ mice per cohort; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

(C) Representative endoscopy images (top row) of distal colonic tumors (arrowhead) on day 72 of the CAC model. Representative immunohistochemical staining for the pan-hematopoietic marker CD45 (middle row) and the macrophage marker F4/80 (bottom row) on adjacent sections of distal colonic tumors from mice of the indicated genotype. Arrows indicate submucosal cell infiltrates. The insets depict higher magnification areas. Scale bars: 200 μm; inset: 30 μm.

(D) Quantification of inflammatory cell infiltrates detected in the submucosa of the sections in (C). Scores are presented as mean ± SEM ($n \geq 3$ mice per cohort; ***$p < 0.001$).

See also Figure S3.

(Figures 3C and 3D) and associated *Tnf* expression, while the expression of other inflammatory cytokines was not altered (Figure S3B). Our results suggest that tumorigenesis depends on IL-11 signaling and correlates with the severity of inflammation and that IL-11 mediates these effects through nonhematopoietic cells.

Sporadic Intestinal Tumorigenesis Requires IL-11 Signaling

The vast majority of sporadic human CRCs arise as a consequence of somatic mutations in components of the Wnt/β-catenin pathway in the absence of chronic inflammatory conditions. To examine the role of cytokines that signal via gp130 receptor

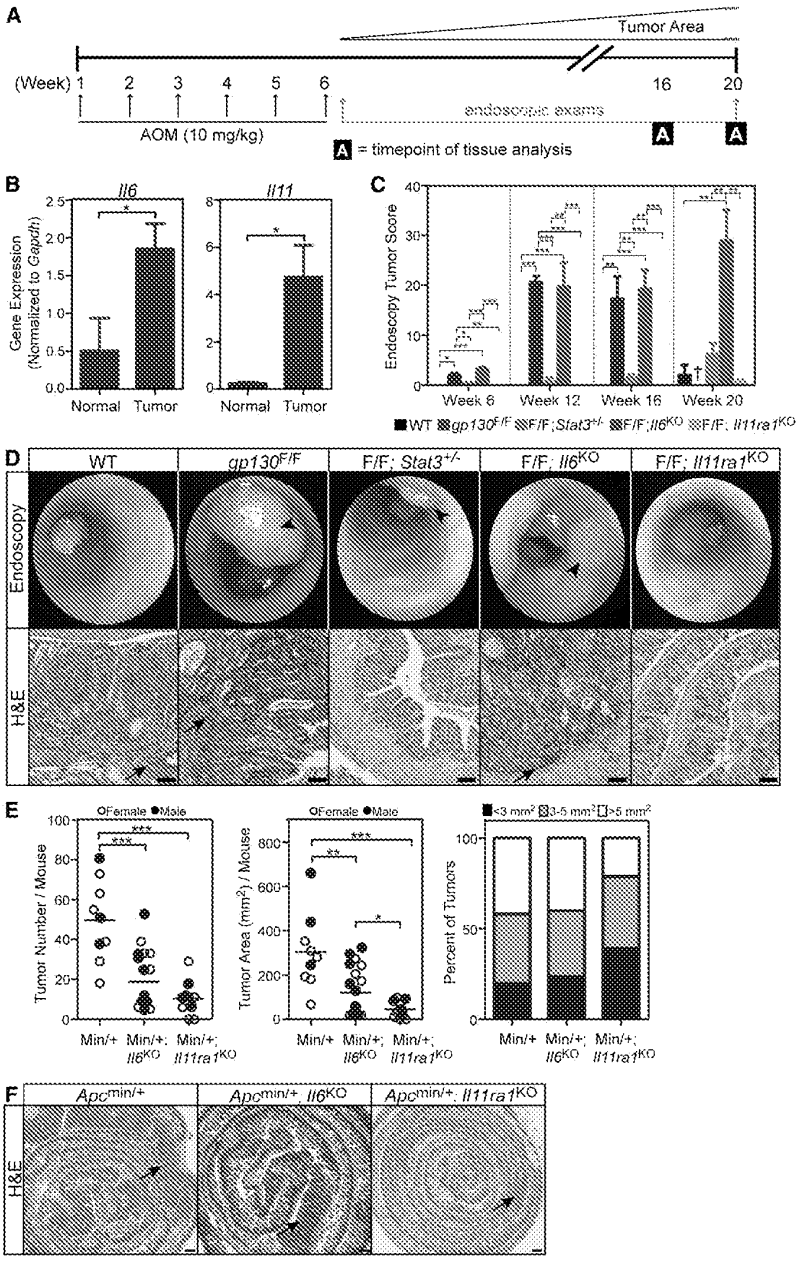


Figure 4. Loss of IL-11/STAT3 Signaling Delays the Onset of Sporadic CRC in Mice

(A) Schematic representation of the sporadic CRC model. The spontaneous formation of distal colonic tumors in mice injected weekly with AOM is monitored by endoscopy.

(B) *Il6* and *Il11* mRNA expression in distal colonic tumor and adjacent nontumor tissue from *gp130*$^{F/F}$ mice collected at week 16 of the sporadic CRC model. Data were normalized to *Gapdh* expression and are shown as mean relative expression (2^dCT) ± SEM ($n \geq 3$ mice per cohort; $^{*}p < 0.05$).

(C) Tumor burden of individual mice of the indicated genotype was scored by endoscopy at the indicated time and is presented as mean ± SEM ($n \geq 4$ mice per cohort; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). †Moribund *gp130*$^{F/F}$ mice that were euthanized by 18 weeks for ethical compliance.

(D) Representative endoscopy images (top row) of distal colonic tumors (arrowhead) at weeks 18–20 of the sporadic CRC model. Representative images of H&E tissue sections (bottom row) with regions of inflammatory cell infiltration (arrows) in the tumor microenvironment. Scale bars: 200 μm.

(E) Colonic tumor burden in individual *Apc*$^{min/+}$ compound mutant mice at 150 days of age. Horizontal lines refer to mean values ($n \geq 9$ mice per cohort; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

(F) Representative images of H&E sections of the distal small intestine from compound mutant *Apc*$^{min/+}$ mice of the indicated genotypes. Scale bars: 200 μm.

See also Figure S4.

complexes in the absence of overt inflammation, we challenged WT and *gp130*$^{F/F}$ mice repeatedly with AOM (Figure 4A). Because the distal colonic tumors of *gp130*$^{F/F}$ mice showed increased *Il6* and *Il11* expression compared to unaffected adjacent tissue (Figure 4B), we carried out serial endoscopy to assess tumor progression in *gp130*$^{F/F}$ mice with impaired IL-6, IL-11, or STAT3 signaling. In this model of the early stages of sporadic CRC observed in humans, the colonic tumor latency in *gp130*$^{F/F}$ mice was reduced to 20 weeks, compared to over 35 weeks in WT controls (Figure 4C). We consistently observed reduced tumor burden in *gp130*$^{F/F}$; *Il11ra1*^{KO} and *gp130*$^{F/F}$;*Stat3*$^{+/-}$ mice, compared to the *gp130*$^{F/F}$ mice (Figures 4C, 4D, and S4A). This coincided with reduced expression of the STAT3 target gene *Socs3* in the remaining small tumors of *gp130*$^{F/F}$;*Il11ra1*^{KO} mice (Figure S4B), where we surmise that other cytokines may account for the residual expression of *Socs3*. Although pan-inflammatory disease is reduced in *gp130*$^{F/F}$;*Il6*^{KO} mice (Ernst et al., 2008) and associated with prolonged survival compared with their *gp130*$^{F/F}$ littermates, the colonic tumor burden was comparable between the two cohorts (Figures 4C and 4D). These observations are consistent with previous findings that gastric tumorigenesis is not the cause of premature death in the *gp130*$^{F/F}$ mice and collectively confirm a dominant role for IL-11 signaling during sporadic CRC.

Given the dependency of AOM-induced tumorigenesis on activating mutations in *Ctnnb1*, we next examined the cellular distribution of β-catenin in neoplastic crypts. As expected, we observed nuclear β-catenin staining, indicative of active Wnt-signaling, in the cells of emerging tumors and the proliferative intestinal stem cell region of crypts of *gp130*$^{F/F}$ and

gp130$^{F/F}$;*Il11ra1*^{KO} mice (Figure S4C). This was supported by comparable expression of the Wnt-target genes *Cd44*, *Axin2*, and *Myc* between mice of the different genotypes and suggests that alteration of IL-11/STAT3 signaling does not alter Wnt-signaling (Figure S4D). Although our AOM-only CRC model lacks the flares of inflammation arising from DSS administration, limiting STAT3 signaling in *gp130*$^{F/F}$;*Il11ra1*^{KO} or *gp130*$^{F/F}$;*Stat3*$^{+/-}$ mice reduced tumor-associated submucosal inflammation (Figure 4D) and expression of markers associated with inflammation (*Cox2*, *Tnf*), invasion (*Mmp9*), and proliferation (*Ccnd1*; Figure S4D).

Our observations suggest that interference with IL-11/STAT3 signaling may be able to limit Wnt/β-catenin-driven intestinal tumorigenesis. To further validate this hypothesis, we next employed the *Apc*$^{min/+}$ mouse model of familial adenomatous polyposis syndrome, where spontaneous intestinal dysplasia develops from loss of heterozygosity of the *Apc* gene (Su et al., 1992). Reminiscent of our findings in the CAC and sporadic CRC AOM-only models (Figures 2 and 4), we observed elevated expression of *Il6* and *Il11* in *Apc*$^{min/+}$ tumors compared to adjacent unaffected tissue (Figures S4E and S4F). We confirmed that genetic ablation of *Il6* reduced tumor numbers and burden (Baltgalvis et al., 2008), and show that tumorigenesis was even further reduced in *Apc*$^{min/+}$;*Il11ra1*^{KO} mice (Figures 4E and 4F). Collectively, these results suggest that IL-11 signaling is required for the development of intestinal tumors that share aberrant activation of the Wnt/β-catenin pathway as an underlying initiating oncogenic event.

IL-11 Signaling Can Be Targeted Therapeutically

We reasoned that a reduction in GI tumor burden following partial genetic inhibition of the IL-11 signaling pathway was required to justify the development of therapeutics against this pathway. To this end, we utilized *gp130*$^{F/F}$ mice as a validated model of spontaneously arising intestinal-type GC. While the gastric tumors that form in the distal antrum of *gp130*$^{F/F}$ mice have abnormally elevated expression of both IL-6 and IL-11 (Figure S5A), the strict dependence of gastric tumor growth on IL-11 signaling (Ernst et al., 2008) makes it an ideal model to test anti-IL-11 therapies. We found that at all stages of gastric tumor development, *gp130*$^{F/F}$ mice lacking one allele of *Stat3* or *Il11ra1* had consistently reduced tumor burden compared to their control *gp130*$^{F/F}$ littermates (Figures S5B and S5C). These results suggest that partial inhibition of the IL-11/STAT3 signaling axis is sufficient to cause a significant reduction in tumor burden.

To test the effects of an antagonist of IL-11 signaling in a model of GI cancer, we systemically treated *gp130*$^{F/F}$ mice with mIL-11 Mutein, a potent IL-11 signaling antagonist that has a 20-fold higher affinity than IL-11 for binding to IL-11Rα (Lee et al., 2008). We found that the IL-11 antagonist reduced pTyr-STAT3 levels in the gastric tumors and adjacent hyperplastic antrum tissue (Figure 5A). We next explored the therapeutic potential of prolonged systemic mIL-11 Mutein administration and found that regardless of the stage of disease, age, and sex of the mice, mIL-11 Mutein treatment significantly reduced overall tumor burden and gastric epithelial hyperplasia (Figures 5B–5D). Successful treatment with different doses of the mIL-11 Mutein for 4 weeks was not associated with weight loss (Figures S5D and S5E) or changes in hematopoietic composition, including platelet counts (Figure S5F), and did not compromise hemostasis (Figures S5G and S5H).

To explore whether mIL-11 Mutein conferred a transient or a long-lasting therapeutic benefit, we randomly assigned *gp130*$^{F/F}$ mice that were treated with mIL-11 Mutein for 4 weeks to either a cohort for immediate analysis or a cohort for a 4-week treatment-free follow-up period. We found that gastric tumor burden in the follow-up cohort was increased compared to the mIL-11 Mutein treatment-only cohort (Figures 5E–5G), suggesting that continuous inhibition of IL-11 signaling is required to block tumors.

To investigate the mechanism by which mIL-11 Mutein reduces tumor burden, we quantified the numbers of PCNA-positive proliferative and Apop-tag-positive apoptotic epithelial cells in the gastric tumors (Figures 6A and S6A). The decrease in PCNA-positive cells observed in mIL-11 Mutein-treated animals coincided with reduced expression of the cell-cycle regulators Cyclin D1, D2, and D3 (Figure 6B). Surprisingly, we found that mIL-11 Mutein treatment was also associated with increased expression of the pro-apoptotic protein BIM, rather than a reduction in the STAT3 target prosurvival protein BCL-2 (Figure 6C). Moreover, mIL-11 Mutein treatment diminished submucosal inflammation, reflected by the reduced infiltration of CD45-positive hematopoietic and F4/80-positive macrophage cells and reduced expression of the inflammatory mediators KC and IL-1β (Figures 6D, S6B, and S6C).

IL-6 and IL-11 Activate Similar Gene Expression Signatures but Target Different Cells

Because both IL-6 and IL-11 are secreted by epithelial and CD45-positive hematopoietic cells in the gastric tissue of *gp130*$^{F/F}$ mice (Figures S6D and S6E), we performed microarray analysis to explore the underlying mechanism for the requirement of IL-11 signaling for gastric tumor promotion. We compared gene expression in gastric tumors of *gp130*$^{F/F}$ mice following a single injection of recombinant human IL-6 or IL-11. The gene expression profiles of the IL-6- and IL-11-treated samples were separately compared to the PBS-vehicle-treated control samples, resulting in two lists of responsive genes (Table S1). Comparison of these lists revealed an extensive overlap of responsive genes between the two cytokines (i.e., *Socs3*, *Vegfa*, *Pim3*, *Reg3b*) with a notable bias for a more profound response to IL-6 (Table S1; Figure S6F). As expected, functional classification analysis showed that genes on either list belong to 13 common functional gene clusters (Figure S6G). However, we also identified several genes that were specifically regulated by only one of the two cytokines (Figure S6F). We found that some of the IL-6-specific genes were associated with immune cells (i.e., *Dusp6, Junb, Cish/Socs1*), while those specific for IL-11 were associated with epithelial activities (i.e., *Spp1/Osteopontin*). The small difference in gene signatures associated with different cellular functions suggested that IL-6 and IL-11 may target different cell populations. To explore this possibility, we adoptively transferred *gp130*$^{F/F}$;*Il11ra1*^{KO} bone marrow into lethally irradiated *gp130*$^{F/F}$ mice with early gastric tumors. We found that the tumor burden in these mice remained similar to that of *gp130*$^{F/F}$ littermates reconstituted with *gp130*$^{F/F}$ bone marrow (Figure 6E). Collectively, our data suggest that IL-6 and IL-11 activate similar gene expression signatures, with gastric

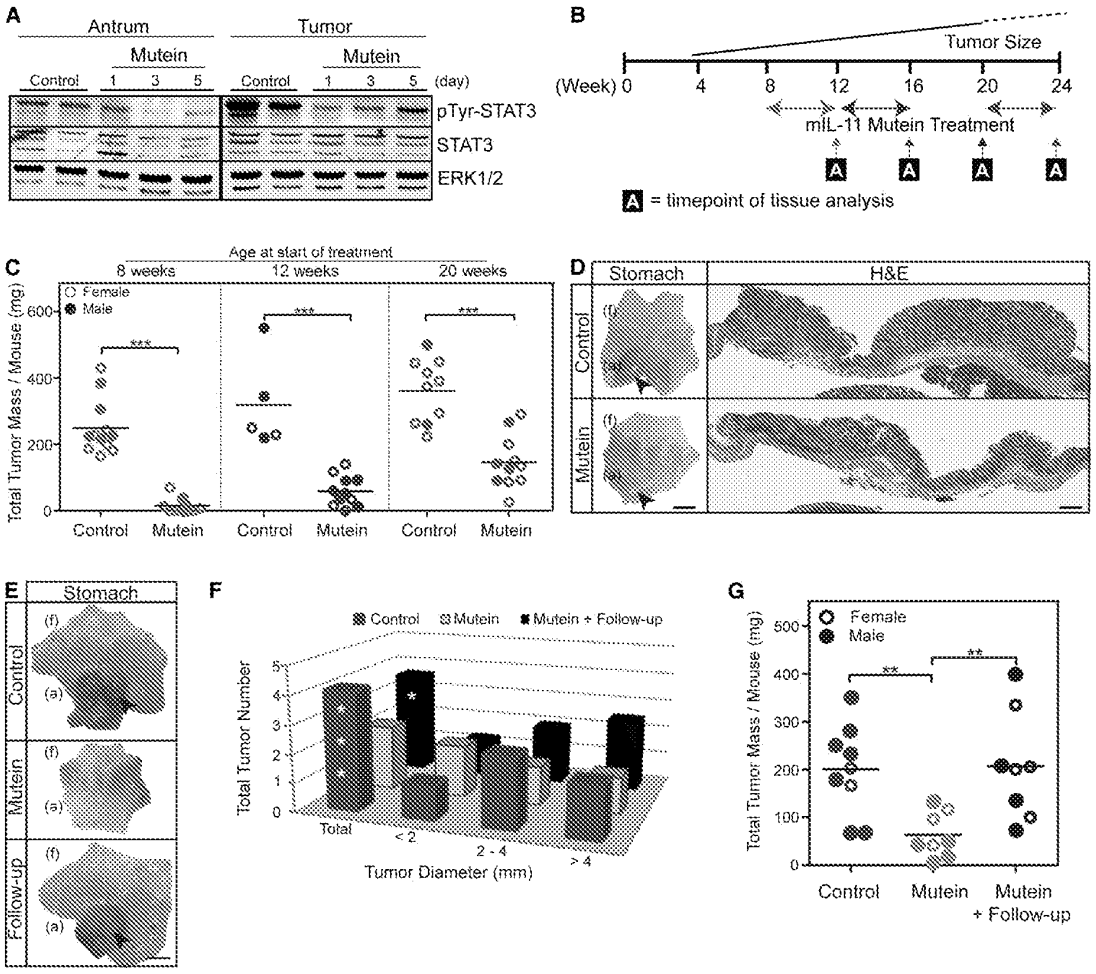


Figure 5. Pharmacologic Inhibition of IL-11 Signaling Inhibits Gastric Tumorigenesis

(A) Representative immunoblot analysis for activated STAT3 (pTyr-STAT3) in gastric antral and tumor tissue from individual 12-week-old *gp130*$^{F/F}$ mice treated with mIL-11 Mutein or a vehicle control on days 1, 3, and 5. ERK1/2 was used as a loading control.

(B) *gp130*$^{F/F}$ mice with established gastric tumors were treated with mIL-11 Mutein or a vehicle control for 4 consecutive weeks. Treatment commenced at 8 (red), 12 (blue), or 20 weeks (green) of age.

(C) Total gastric tumor mass of individual control- and mIL-11 Mutein-treated *gp130*$^{F/F}$ mice of the treatment age group indicated in (B). Horizontal lines refer to mean values ($n \geq 5$ mice per cohort; ***$p < 0.001$).

(D) Representative whole mounts of stomachs (left) and corresponding H&E longitudinal sections (right) from 16-week-old control and mIL-11 Mutein-treated *gp130*$^{F/F}$ mice. Stomachs were opened along the greater curvature and pinned-out with the lumen facing the viewer (f = fundus, a = antrum). Scale bars: 6 mm (whole mount); 500 μm (H&E).

(E–G) Sixteen-week-old *gp130*$^{F/F}$ mice were analyzed after 4 consecutive weeks of mIL-11 Mutein treatment or after an additional 4-week treatment-free follow-up period. Representative whole mounts of stomachs of *gp130*$^{F/F}$ mice of the indicated treatment group (E); f, fundus; a, antrum; scale bar: 6 mm. In each mouse, individual tumors were classified according to their size (F) and the combined mass of resected tumors was determined (G). Horizontal lines and histograms refer to mean values ($n \geq 8$ per mice cohort; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

See also Figure S5.

tumorigenesis likely to arise from cells in the glandular epithelium that may be biased toward IL-11 responsiveness.

mIL-11 Mutein Therapy Suppresses CAC Tumor Progression

Having demonstrated that IL-11 signaling can be therapeutically targeted in the *gp130*$^{F/F}$ model of spontaneous GC (Figure 5), we next examined whether mIL-11 Mutein could confer a therapeutic benefit in the absence of predetermined mutations in *gp130*$^{F/F}$ mice. Therefore, we treated WT mice with established CAC tumors for 4 weeks with mIL-11 Mutein (Figure S7A). Serial endoscopy revealed that mIL-11 Mutein treatment led to a cytostatic effect on existing colonic tumors (Figure 7A), which was confirmed at autopsy by a significant reduction in tumor

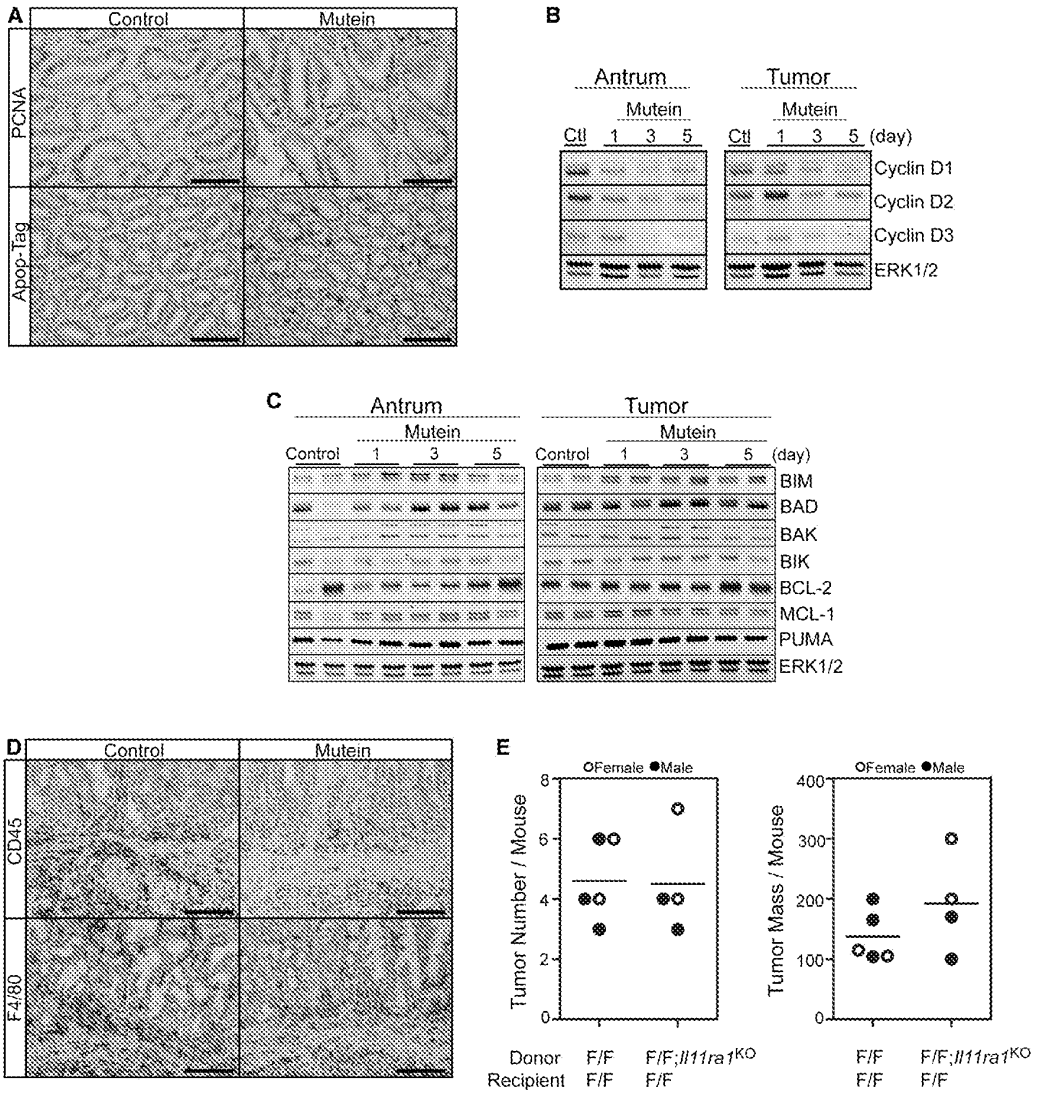


Figure 6. Mutein Treatment Reduces Tumor Cell Proliferation and Triggers Cell Death

(A) Representative immunohistochemical staining for proliferation (PCNA) and cell death (Apop-Tag) markers on adjacent gastric tumor sections from control- and mIL-11 Mutein-treated *gp130*$^{F/F}$ mice at 12 weeks of age. Scale: 200 μm.

(B and C) Immunoblot analysis of gastric antral and tumor tissue from individual 12-week-old *gp130*$^{F/F}$ mice treated with mIL-11 Mutein or a vehicle control (Ctl) on days 1, 3, and 5 and analyzed for the expression of cyclin (B) or apoptosis-related proteins (C). ERK1/2 was used as a loading control.

(D) Representative immunohistochemical stainings for the pan-hematopoietic marker CD45 and the macrophage marker F4/80 on adjacent gastric tumor sections from control and mIL-11 Mutein-treated *gp130*$^{F/F}$ mice at 12 weeks of age. Scale bars: 200 μm.

(E) Total gastric tumor numbers (left) and mass (right) of *gp130*$^{F/F}$ bone marrow chimeras at 14 weeks of age. Horizontal lines refer to mean values ($n \geq 4$ mice per cohort).

See also Figure S6 and Table S1.

multiplicity and size compared to vehicle-treated animals (Figures 7B and 7C). We also confirmed that mIL-11 Mutein significantly alleviated CAC-induced tumor burden in *gp130*$^{F/F}$ mice (Figures S7B and S7C). Importantly, we show that in *gp130*$^{F/F}$ mice, mIL-11 Mutein treatment reduced neoplastic colonic epithelial proliferation and increased apoptosis (Figures S7D and S7E). Interestingly, *gp130*$^{F/F}$ mice display elevated colonic tumor BCL-2 expression compared to WT mice (Figure 2F), which was not altered following Mutein treatment (Figure S7F). Similar to our results from the GC model (Figure 6), we attribute the therapeutic benefit of mIL-11 Mutein in colonic tumors to a reduction in tumor-associated STAT3 activation and increased expression of pro-apoptotic BIM (Figures 7D and S7F). Taken together, these results indicate that inhibition of IL-11 signaling

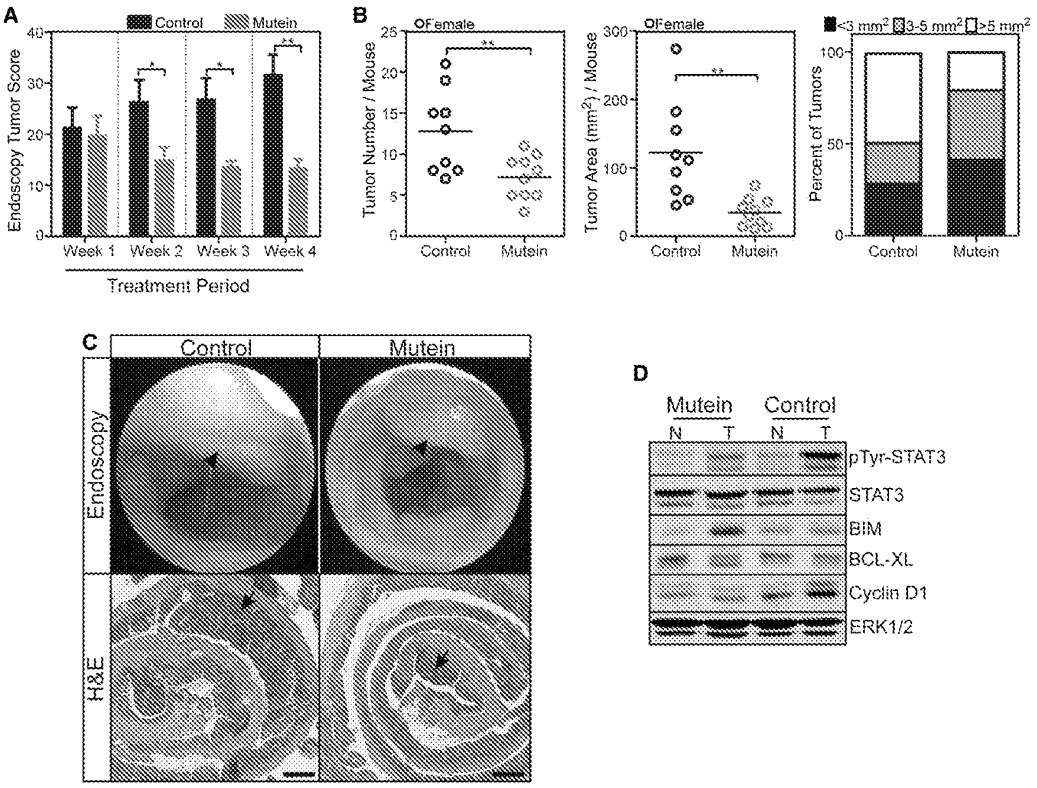


Figure 7. Therapeutic Inhibition of IL-11 Signaling Reduces Tumor Burden in CAC-Challenged WT Mice

(A) CAC-challenged WT mice were treated with mIL-11 Mutein for 4 consecutive weeks commencing on day 46 of the CAC model (refer to Figure S7A). Tumor burden was scored by serial endoscopy at the indicated time points and is presented as mean ± SEM ($n \geq 4$ mice per cohort; $^{*}p < 0.05$, $^{**}p < 0.01$).

(B) Colonic tumor burden in individual control and mIL-11 Mutein-treated WT mice at autopsy on day 72. Horizontal lines refer to mean values ($n \geq 9$ mice per cohort; $^{**}p < 0.01$).

(C) Representative endoscopy images (top row) of distal colonic tumors (arrowhead) and corresponding H&E tissue sections (bottom row) with tumors and associated inflammatory cells (arrows) in control and mIL-11 Mutein-treated WT mice at day 72 of the CAC protocol. Scale bars: 200 μm.

(D) Representative immunoblot analysis of distal colonic tumor (T) and adjacent nontumor (N) tissue from control and mIL-11 Mutein-treated WT mice collected on day 72 of the CAC model. ERK1/2 was used as a loading control.

See also Figure S7.

promotes anti-tumor effects in a well-established model of human CRC.

mIL-11 Mutein Therapy Slows the Growth of Human GI Cancer Cells in Xenografts

To investigate whether mIL-11 Mutein could also impede the growth of human GI cancers, we used two representative human GI cancer derived cell lines, DLD1 and MKN28. DLD1 cells harbor mutations in *APC, PIK3CA, TP53*, and *KRAS*, which are frequently observed in CRC (Trainer et al., 1988), whereas MKN28 cells have mutations in *TP53*, prevalent in GC (Matozaki et al., 1992). In response to stimulation with recombinant human IL-11, both cell lines displayed dose-dependent activation of STAT3 (pTyr-STAT3 staining; Figures 8A and S8A). Because mIL-11 Mutein cross-reacts with the human IL-11Rα, we pretreated these cell lines with mIL-11 Mutein and found a dose-dependent inhibition of hIL-11-mediated STAT3 activation (Figures 8B and S8B). Given that we observed increased STAT3 activation at the invasive front of human tumors (Figure 1C) and because IL-11 has been implicated in the invasion and metastasis of epithelial cancer cells (Calon et al., 2012; Lay et al., 2012), we next examined whether mIL-11 Mutein treatment could inhibit the invasive potential of human GI cancer cells in vitro. We found that recombinant hIL-11 promoted cell migration in a transwell assay and that inhibition of IL-11 signaling by pretreatment with mIL-11 Mutein blocked the invasive capacity of DLD1 and MKN28 cells (Figures 8C and S8C). To assess whether the growth of human cancer cells in vivo was susceptible to inhibiting the activity of IL-11 that is produced by both tumor cells and the surrounding stroma (Figure S8D), we established subcutaneous xenografts of DLD1 tumor cells in immune-deficient BALB/c nude mice. Once tumors became palpable, we treated mice with mIL-11 Mutein for 4 consecutive weeks. We observed significantly reduced tumor growth in mIL-11 Mutein-treated animals compared to vehicle-treated controls, resulting in an ~50% decrease in tumor mass at autopsy (Figures 8D and 8E). Immunohistochemical analysis of the tumors revealed a significant reduction of activated STAT3

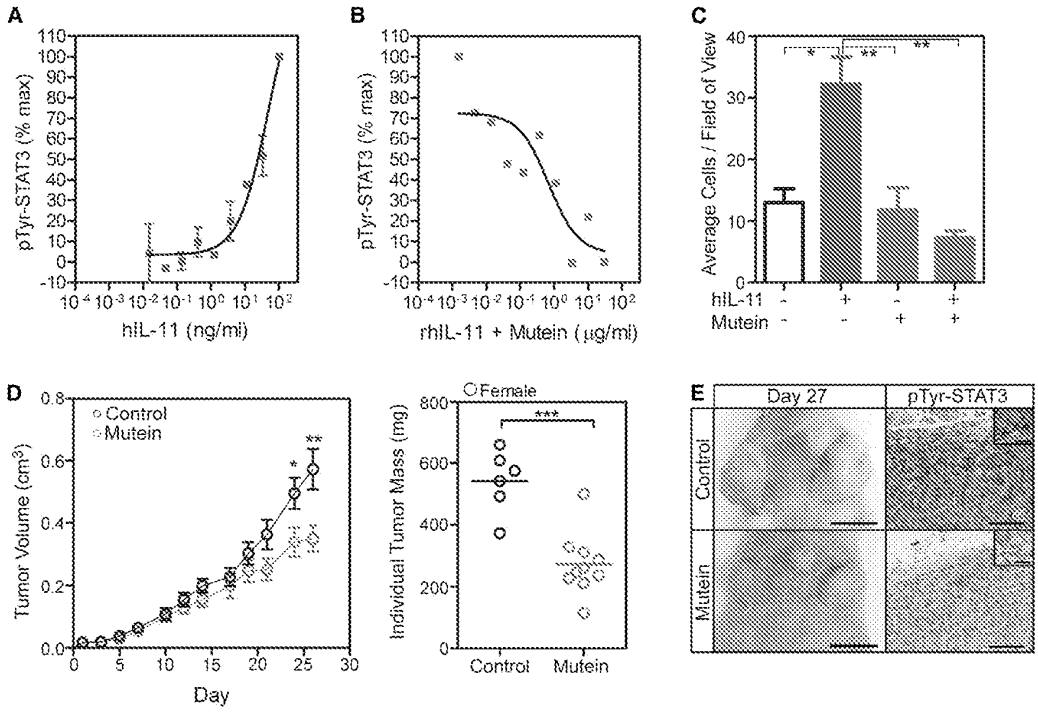


Figure 8. Therapeutic Inhibition of IL-11 Signaling Reduces the Growth of Human CRC Cells in Immune-Deficient Mice

(A and B) pTyr-STAT3 in DLD1 cells stimulated with increasing concentrations of recombinant hIL-11 (A) or stimulated with 20 ng/ml of hIL-11 following pretreatment with increasing concentrations of mIL-11 Mutein (B). pTyr-STAT3 was determined by flow cytometry. Data in (A) are means ± SEM.

(C) DLD1 cells pretreated with mIL-11 Mutein (100 μg/ml) for 60 min were combined with recombinant hIL-11 and Matrigel as indicated. The mixture was added to the top chambers of transwell plates (2×10^5 cells/well) with IL-11-deficient medium in the bottom chambers. Cells that migrated to the bottom chamber were enumerated 24 hr later. Results represent mean ± SEM (*$p < 0.05$, **$p < 0.01$).

(D) BALB/c nude mice were injected subcutaneously with 5×10^6 DLD1 cells on contralateral sides. When palpable tumors had formed 3 days later, the mice were treated three times per week over 24 days with mIL-11 Mutein (10 mg/kg) or a vehicle control. Tumor volumes were calculated from serial caliper measurements, and the total tumor mass of each individual tumor was determined at autopsy. Horizontal lines refer to mean values. Data are means ± SEM ($n \geq 3$ mice per treatment group; *$p < 0.05$, **$p < 0.01$).

(E) Representative photographs of DLD1 xenograft tumors on day 27 (left; scale bars: 11 mm) and corresponding immunohistochemical stains for pTyr-STAT3 (right; scale bars: 100 μm). Insets represent higher magnifications (scale bars: 25 μm).

See also Figure S8.

(pTyr-STAT3 staining; Figure 8E) consistent with our proposed role for IL-11-mediated STAT3 signaling in the promotion of GI cancers. Collectively, our data confirm that therapeutic inhibition of IL-11 signaling inhibits the progression of human GI tumors.

DISCUSSION

Emerging therapeutic strategies for epithelial cancers have explored cytokine inhibition as an alternative to pharmacologic targeting of signal transducing kinases and intracellular transcription factors. A major focus has been placed on cytokines that suppress tumor cell apoptosis through activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), including IL-1β and tumor necrosis factor-α (Greten et al., 2004; Popivanova et al., 2008). Meanwhile, STAT3-activating cytokines are also attracting therapeutic interest, due to the prevalence of STAT3 activation in many types of human cancers, and the capacity of STAT3 to promote epithelial tumor progression and to suppress the host's antitumor immune response (Becker et al., 2004; Bollrath et al., 2009; Yu et al., 2007). In particular, antibodies that neutralize IL-6 (i.e., siltuximab) or block IL-6Rα (i.e., tocilizumab) are in clinical trials for ovarian, prostate, and renal cancers (Guo et al., 2010; Karkera et al., 2011). Our data argue that despite its overlapping expression with IL-6, IL-11 is the dominant STAT3-activating cytokine required for the progression of GI cancers.

Although the contribution of inflammatory cells to GI tumorigenesis is well established (Rutter et al., 2004), the beneficial impact of interfering with specific hematopoietic cell types appears to vary between different types of cancer. Genetic ablation of T and B cells (Boulard et al., 2012), macrophages (Oguma et al., 2008), mast cells (Gounaris et al., 2007), and components of the TLR/Myd88-signaling cascade (Rakoff-Nahoum and Medzhitov, 2007; Tye et al., 2012) reduced GI tumor burden in mice. Here, we show that the progression of GI tumors is not influenced by the capacity of hematopoietic cells to respond to IL-11, but instead requires the response of nonhematopoietic cells, such as the neoplastic epithelium itself. However, our

analysis does not preclude the possibility that bone marrow-derived cells are a critical source for IL-11, which in turn may account for the capacity of myeloid cells to promote invasiveness of epithelial tumors (Coussens et al., 2000). Consistent with this, we observed reduced IL-11 expression in NF-κB signaling-defective myeloid cells, which suppressed invasion of AOM-induced tumors in *Tp53* knockout mice (Schwitalla et al., 2013).

At this stage, it is not clear if the subtle difference in gene expression signatures observed between tumors from IL-6- and IL-11-challenged $gp130^{F/F}$ mice accounts for IL-11-dependent tumor development or whether tumors arise from a distinct (epithelial) cell population that selectively expresses IL-11Rα, but not the IL-6Rα. We favor the latter scenario, because the administration of Hyper-IL-6, a designer cytokine comprised of IL-6 fused to the IL-6Rα cytoplasmic tail, bypassed the requirement for membrane bound IL-11Rα and increased CAC tumor burden is an outcome not observed following IL-6 administration (Grivennikov et al., 2009). Consistent with an IL-6 "trans-signaling" mechanism, transgenic mice expressing sgp130-Fc, which competes with membrane gp130 for binding of IL-6 complexed to the cleaved IL-6Rα cytosolic domain, are less susceptible to CAC-induced tumorigenesis (Becker et al., 2004; Matsumoto et al., 2010). Although "trans-signaling" may also expand the function of IL-11 to IL-11Rα-deficient tumor cells, cleavage of the extracellular domain of IL-11Rα has not been observed in vivo. Given that IL-11 expression is also augmented in response to oncogenic RAS signaling (Shin et al., 2012), our results suggest that inhibition of IL-11 signaling may restore responsiveness to epidermal growth factor receptor (EGFR) therapy in the broad range of epithelial cancers that are characterized by excessive EGFR expression. This seems plausible, because cetuximab and other EGFR inhibitors show efficacy for the treatment of cancers without K-RAS mutations (Quesnelle et al., 2007), while resistance to EGFR inhibition can be overcome by simultaneous STAT3 suppression (Sen et al., 2012).

We predict that the reliance of the GI tract on IL-11/STAT3 signaling for tumor progression evolved from an intestinal repair mechanism to provide protection against the continuous inflammation caused by mechanical abrasion of the epithelium, which is observed in STAT3 signaling-deficient $gp130^{\Delta Stat}$ mice (Tebbutt et al., 2002). By exposing the injured epithelium to platelets, and other IL-11-producing CD45-positive cells, the mucosal cell layer can be regenerated from IL-11Rα expressing mucosal (stem) cells, in part through a STAT3-dependent mechanism. In the newly emerging epithelium, STAT3 would not only engage anti-apoptotic and proliferative gene programs, but also simultaneously amplify IL-11 expression (Boerma et al., 2007; Deutscher et al., 2006; Ernst et al., 2008; Gibson et al., 2010; Orazi et al., 1996). Incidentally, inhibition of IL-11 signaling increased expression of the pro-apoptotic protein BIM in a manner akin to that observed in response to therapeutic inhibition of the STAT3-target VEGF-A (Naik et al., 2011). The functional link between the epithelial repair response and the extent of local inflammation therefore requires an IL-6 family cytokine with a tissue specific response like IL-11, rather than the broad systemic activity elicited by IL-6 that can also exacerbate intestinal inflammation. Indeed, in the absence of overt colitis, the inflammatory microenvironment associated with sporadic *Apc* or *Tp53* mutations confers a growth-promoting advantage to adenomas (Grivennikov et al., 2012; Schwitalla et al., 2013). IL-11 may also facilitate the survival of advanced cancers with multiple mutations and increase their metastatic capacity (Calon et al., 2012). The latter effect can be further exacerbated by CAFs, which are an additional source of IL-11 (Calon et al., 2012) as well as by tumor hypoxia that is associated with increased IL-11 production (Onnis et al., 2013).

Direct therapeutic targeting of STAT3 has proven difficult, although inhibition of upstream activators, including JAK2, has shown more promise (Hedvat et al., 2009). Unfortunately, systemic inhibition of STAT3 or JAK2 in mice or humans (Ernst et al., 2008; Santos et al., 2010) is often associated with thrombocytopenia. Here, we show that a peptide-based IL-11 antagonist is well tolerated with no adverse effects on platelet numbers, despite the capacity of IL-11 to stimulate megakaryopoiesis (Musashi et al., 1991). These observations are consistent with the phenotype of mice lacking the IL-11 receptor α chain, which exhibit normal hematopoiesis at steady state and during response to chemoablative or hemolytic stress (Nandurkar et al., 1997). However, our results bring into question the clinical use of recombinant IL-11 for the treatment of thrombocytopenia in patients undergoing chemotherapy or to reconstitute epithelial barrier integrity in patients with inflammatory bowel disease (Cantor et al., 2003; Danese, 2012).

By identifying IL-11 as a functionally dominant inducer of neoplastic STAT3 activity in the GI epithelium, our studies establish a molecular entity that can be readily targeted by pharmacologic agents. Therapeutic interference with IL-11 signaling, therefore, could potentially selectively suppress the STAT3-associated cancer hallmark capabilities that collectively promote disease progression of some of the most prevalent epithelial cancer types in humans. It remains to be established whether the therapeutic inhibition of IL-11/STAT3 signaling in turn exposes compensatory pathways in tumor cells that can be further exploited to induce tumor cell death.

EXPERIMENTAL PROCEDURES

Clinical Material

All human tissues were obtained with informed patient consent and analyzed in accordance with approval from the ethics committee of the Technical University of Munich, the Peter MaCallum Cancer Center, and the Melbourne Health Human Ethics Committee (2010.154; 2012.25). Tumor and adjacent normal (noncancerous) tissues were analyzed.

Mice and Treatments

All animal procedures were approved and conducted in accordance with the Animal Ethics Committee of the Ludwig Institute for Cancer Research and the Walter and Eliza Hall Institute, Australia. Mice lacking functional alleles for IL-11Rα ($Il11ra1^{KO}$) and IL-6 ($Il6^{KO}$), homozygous for the gp130(Y757F) knockin mutation ($gp130^{F/F}$), or heterozygous for *Apc* ($Apc^{min/+}$) have been described previously (Ernst et al., 2008; Jenkins et al., 2005; Moser et al., 1990). The $Apc^{min/+}$ compound mutant mice were on a C57Bl/6 genetic background, whereas others were on a 129/sv x C57Bl/6 genetic background. Littermate controls were used for comparison when possible. BALB/c nude animals were purchased from the Australian Research Services. To minimize variation in gut microflora, all animals were bred in the same room and housed on the same rack in a specific pathogen-free barrier facility at the Ludwig Institute for Cancer Research, Australia.

CAC was induced in 129/sv × C57Bl/6 mice by intraperitoneal injection of AOM (10 mg/kg; Sigma). One week later, animals were provided drinking water

ad libitum containing 2% DSS (w/v; MP Biomedicals, molecular weight 35,000–50, 000 kDa) for 5 days, followed by 2 weeks of normal drinking water, which was repeated for two or three cycles as indicated. For CRC, animals received intraperitoneal injections of AOM (10 mg/kg) once weekly over 6 consecutive weeks. Tumor onset and progression in the distal colon was monitored by endoscopy as described previously (Becker et al., 2006).

Xenografts were established in 6-week-old nude BALB/c mice by subcutaneous injection of 5×10^6 DLD1 cells. Tumor volume was measured every second day with calipers and calculated using the formula: 0.52*(Tumor Length*(Tumor Width)^2)/1,000. For therapeutic mIL-11 Mutein treatments, mice received intraperitoneal injections three times a week over 4 consecutive weeks with 40 mg/kg mIL-11-Mutein or a PEG-vehicle control (unless otherwise indicated). The affinity, specificity, and bioactivity of mIL-11-Mutein has been described previously (Lee et al., 2008).

Statistics

All data are representative of at least two independent experiments. Comparisons between values from two groups were performed using Student's t tests (two tailed) and multiple groups by ANOVA (Bonferroni post-hoc): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Additional experimental information is available in the Supplemental Experimental Procedures.

ACCESSION NUMBER

The NCBI Gene Expression Omnibus repository number for the complete array data presented in this paper is GSE43800.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, eight figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2013.06.017.

ACKNOWLEDGMENTS

We thank R. Hughan, C. Dijkstra, C. Nowell, J. Corbin, and the LICR Animal Facility and Histology staff for excellent technical assistance. We thank A. Strasser, T. Rowe, M. Griffin, and B. Klopcic for critical review of the manuscript and P. Waring and M. Christie for pathological assessment of the mouse and human histology, respectively. A cohort of the de-identified human GC tissue was provided by the Victorian Cancer Biobank. This work was made possible through Victorian State Government Operational Infrastructure Support and Australian Government NHMRC project grant 1008614 and program grants 487922 and 1016647. T.L.P. also received funding from the Cancer Australia priority driven research scheme, Cure Cancer Australia grant 603121. P.K.Z. was supported by a doctoral fellowship from the Deutsche Krebshilfe (110114). M.E., B.T.K., and J.K.H. are Senior Research Fellows of the NHMRC. N.J.W., K.M.E., A.N., and B.S.M. are employees of CSL, Ltd., and T.L.P. and M.E. received research support from CSL, Ltd.

Received: February 1, 2013
Revised: May 17, 2013
Accepted: June 27, 2013
Published: August 12, 2013

REFERENCES

Baltgalvis, K.A., Berger, F.G., Pena, M.M., Davis, J.M., Muga, S.J., and Carson, J.A. (2008). Interleukin-6 and cachexia in ApcMin/+ mice. Am. J. Physiol. Regul. Integr. Comp. Physiol. *294*, R393–R401.

Becker, C., Fantini, M.C., Schramm, C., Lehr, H.A., Wirtz, S., Nikolaev, A., Burg, J., Strand, S., Kiesslich, R., Huber, S., et al. (2004). TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. Immunity *21*, 491–501.

Becker, C., Fantini, M.C., and Neurath, M.F. (2006). High resolution colonoscopy in live mice. Nat. Protoc. *1*, 2900–2904.

Boerma, M., Wang, J., Burnett, A.F., Santin, A.D., Roman, J.J., and Hauer-Jensen, M. (2007). Local administration of interleukin-11 ameliorates intestinal radiation injury in rats. Cancer Res. *67*, 9501–9506.

Bollrath, J., Phesse, T.J., von Burstin, V.A., Putoczki, T., Bennecke, M., Bateman, T., Nebelsiek, T., Lundgren-May, T., Canli, O., Schwitalla, S., et al. (2009). gp130-mediated Stat3 activation in enterocytes regulates cell survival and cell-cycle progression during colitis-associated tumorigenesis. Cancer Cell *15*, 91–102.

Boulard, O., Kirchberger, S., Royston, D.J., Maloy, K.J., and Powrie, F.M. (2012). Identification of a genetic locus controlling bacteria-driven colitis and associated cancer through effects on innate inflammation. J. Exp. Med. *209*, 1309–1324.

Calon, A., Espinet, E., Palomo-Ponce, S., Tauriello, D.V., Iglesias, M., Céspedes, M.V., Sevillano, M., Nadal, C., Jung, P., Zhang, X.H., et al. (2012). Dependency of colorectal cancer on a TGF-β-driven program in stromal cells for metastasis initiation. Cancer Cell *22*, 571–584.

Cantor, S.B., Elting, L.S., Hudson, D.V., Jr., and Rubenstein, E.B. (2003). Pharmacoeconomic analysis of oprelvekin (recombinant human interleukin-11) for secondary prophylaxis of thrombocytopenia in solid tumor patients receiving chemotherapy. Cancer *97*, 3099–3106.

Casanova, J.L., Holland, S.M., and Notarangelo, L.D. (2012). Inborn errors of human JAKs and STATs. Immunity *36*, 515–528.

Coussens, L.M., Tinkle, C.L., Hanahan, D., and Werb, Z. (2000). MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell *103*, 481–490.

Danese, S. (2012). New therapies for inflammatory bowel disease: from the bench to the bedside. Gut *61*, 918–932.

Deng, J.Y., Sun, D., Liu, X.Y., Pan, Y., and Liang, H. (2010). STAT-3 correlates with lymph node metastasis and cell survival in gastric cancer. World J. Gastroenterol. *16*, 5380–5387.

Deutscher, N., Bataille, F., Hausmann, M., Kiessling, S., Muller-Newen, G., Leeb, S.N., Herfarth, H., Heinrich, P.C., Schölmerich, J., and Rogler, G. (2006). Functional expression of the interleukin-11 receptor alpha-chain in normal colonic epithelium and colon cancer. Int. J. Colorectal Dis. *21*, 573–581.

Egeblad, M., Nakasone, E.S., and Werb, Z. (2010). Tumors as organs: complex tissues that interface with the entire organism. Dev. Cell *18*, 884–901.

Ernst, M., Najdovska, M., Grail, D., Lundgren-May, T., Buchert, M., Tye, H., Matthews, V.B., Armes, J., Bhathal, P.S., Hughes, N.R., et al. (2008). STAT3 and STAT1 mediate IL-11-dependent and inflammation-associated gastric tumorigenesis in gp130 receptor mutant mice. J. Clin. Invest. *118*, 1727–1738.

Esfandi, F., Mohammadzadeh Ghobadloo, S., and Basati, G. (2006). Interleukin-6 level in patients with colorectal cancer. Cancer Lett. *244*, 76–78.

Gao, S.P., Mark, K.G., Leslie, K., Pao, W., Motoi, N., Gerald, W.L., Travis, W.D., Bornmann, W., Veach, D., Clarkson, B., and Bromberg, J.F. (2007). Mutations in the EGFR kinase domain mediate STAT3 activation via IL-6 production in human lung adenocarcinomas. J. Clin. Invest. *117*, 3846–3856.

Gibson, D.L., Montero, M., Ropeleski, M.J., Bergstrom, K.S., Ma, C., Ghosh, S., Merkens, H., Huang, J., Månsson, L.E., Sham, H.P., et al. (2010). Interleukin-11 reduces TLR4-induced colitis in TLR2-deficient mice and restores intestinal STAT3 signaling. Gastroenterology *139*, 1277–1288.

Gounaris, E., Erdman, S.E., Restaino, C., Gurish, M.F., Friend, D.S., Gounari, F., Lee, D.M., Zhang, G., Glickman, J.N., Shin, K., et al. (2007). Mast cells are an essential hematopoietic component for polyp development. Proc. Natl. Acad. Sci. USA *104*, 19977–19982.

Greten, F.R., Eckmann, L., Greten, T.F., Park, J.M., Li, Z.W., Egan, L.J., Kagnoff, M.F., and Karin, M. (2004). IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell *118*, 285–296.

Grivennikov, S., Karin, E., Terzic, J., Mucida, D., Yu, G.Y., Vallabhapurapu, S., Scheller, J., Rose-John, S., Cheroutre, H., Eckmann, L., and Karin, M. (2009). IL-6 and Stat3 are required for survival of intestinal epithelial cells and development of colitis-associated cancer. Cancer Cell *15*, 103–113.

Grivennikov, S.I., Wang, K., Mucida, D., Stewart, C.A., Schnabl, B., Jauch, D., Taniguchi, K., Yu, G.Y., Osterreicher, C.H., Hung, K.E., et al. (2012).

Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. Nature *491*, 254–258.

Guo, Y., Nemeth, J., O'Brien, C., Susa, M., Liu, X., Zhang, Z., Choy, E., Mankin, H., Hornicek, F., and Duan, Z. (2010). Effects of siltuximab on the IL-6-induced signaling pathway in ovarian cancer. Clin. Cancer Res. *16*, 5759–5769.

Guo, Y., Xu, F., Lu, T., Duan, Z., and Zhang, Z. (2012). Interleukin-6 signaling pathway in targeted therapy for cancer. Cancer Treat. Rev. *38*, 904–910.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646–674.

Hanahan, D., and Coussens, L.M. (2012). Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell *21*, 309–322.

He, B., You, L., Uematsu, K., Zang, K., Xu, Z., Lee, A.Y., Costello, J.F., McCormick, F., and Jablons, D.M. (2003). SOCS-3 is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer. Proc. Natl. Acad. Sci. USA *100*, 14133–14138.

Hedvat, M., Huszar, D., Herrmann, A., Gozgit, J.M., Schroeder, A., Sheehy, A., Buettner, R., Proia, D., Kowolik, C.M., Xin, H., et al. (2009). The JAK2 inhibitor AZD1480 potently blocks Stat3 signaling and oncogenesis in solid tumors. Cancer Cell *16*, 487–497.

Heinrich, P.C., Castell, J.V., and Andus, T. (1990). Interleukin-6 and the acute phase response. Biochem. J. *265*, 621–636.

Heinrich, P.C., Behrmann, I., Müller-Newen, G., Schaper, F., and Graeve, L. (1998). Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem. J. *334*, 297–314.

Jenkins, B.J., Grail, D., Nheu, T., Najdovska, M., Wang, B., Waring, P., Inglese, M., McLoughlin, R.M., Jones, S.A., Topley, N., et al. (2005). Hyperactivation of Stat3 in gp130 mutant mice promotes gastric hyperproliferation and desensitizes TGF-beta signaling. Nat. Med. *11*, 845–852.

Karkera, J., Steiner, H., Li, W., Skradski, V., Moser, P.L., Riethdorf, S., Reddy, M., Puchalski, T., Safer, K., Prabhakar, U., et al. (2011). The anti-interleukin-6 antibody siltuximab down-regulates genes implicated in tumorigenesis in prostate cancer patients from a phase I study. Prostate *71*, 1455–1465.

Kim, D.Y., Cha, S.T., Ahn, D.H., Kang, H.Y., Kwon, C.I., Ko, K.H., Hwang, S.G., Park, P.W., Rim, K.S., and Hong, S.P. (2009). STAT3 expression in gastric cancer indicates a poor prognosis. J. Gastroenterol. Hepatol. *24*, 646–651.

Lay, V., Yap, J., Sonderegger, S., and Dimitriadis, E. (2012). Interleukin 11 regulates endometrial cancer cell adhesion and migration via STAT3. Int. J. Oncol. *41*, 759–764.

Lee, C.G., Hartl, D., Matsuura, H., Dunlop, F.M., Scotney, P.D., Fabri, L.J., Nash, A.D., Chen, N.Y., Tang, C.Y., Chen, Q., et al. (2008). Endogenous IL-11 signaling is essential in Th2- and IL-13-induced inflammation and mucus production. Am. J. Respir. Cell Mol. Biol. *39*, 739–746.

Matozaki, T., Sakamoto, C., Matsuda, K., Suzuki, T., Konda, Y., Nakano, O., Wada, K., Uchida, T., Nishisaki, H., Nagao, M., et al. (1992). Missense mutations and a deletion of the p53 gene in human gastric cancer. Biochem. Biophys. Res. Commun. *182*, 215–223.

Matsumoto, S., Hara, T., Mitsuyama, K., Yamamoto, M., Tsuruta, O., Sata, M., Scheller, J., Rose-John, S., Kado, S., and Takada, T. (2010). Essential roles of IL-6 trans-signaling in colonic epithelial cells, induced by the IL-6/soluble-IL-6 receptor derived from lamina propria macrophages, on the development of colitis-associated premalignant cancer in a murine model. J. Immunol. *184*, 1543–1551.

Matsuo, K., Oka, M., Murase, K., Soda, H., Isomoto, H., Takeshima, F., Mizuta, Y., Murata, I., and Kohno, S. (2003). Expression of interleukin 6 and its receptor in human gastric and colorectal cancers. J. Int. Med. Res. *31*, 69–75.

Morikawa, T., Baba, Y., Yamauchi, M., Kuchiba, A., Nosho, K., Shima, K., Tanaka, N., Huttenhower, C., Frank, D.A., Fuchs, C.S., and Ogino, S. (2011). STAT3 expression, molecular features, inflammation patterns, and prognosis in a database of 724 colorectal cancers. Clin. Cancer Res. *17*, 1452–1462.

Moser, A.R., Pitot, H.C., and Dove, W.F. (1990). A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science *247*, 322–324.

Musashi, M., Clark, S.C., Sudo, T., Urdal, D.L., and Ogawa, M. (1991). Synergistic interactions between interleukin-11 and interleukin-4 in support of proliferation of primitive hematopoietic progenitors of mice. Blood *78*, 1448–1451.

Naik, E., O'Reilly, L.A., Asselin-Labat, M.L., Merino, D., Lin, A., Cook, M., Coultas, L., Bouillet, P., Adams, J.M., and Strasser, A. (2011). Destruction of tumor vasculature and abated tumor growth upon VEGF blockade is driven by proapoptotic protein Bim in endothelial cells. J. Exp. Med. *208*, 1351–1358.

Nakayama, T., Yoshizaki, A., Izumida, S., Suehiro, T., Miura, S., Uemura, T., Yakata, Y., Shichijo, K., Yamashita, S., and Sekin, I. (2007). Expression of interleukin-11 (IL-11) and IL-11 receptor alpha in human gastric carcinoma and IL-11 upregulates the invasive activity of human gastric carcinoma cells. Int. J. Oncol. *30*, 825–833.

Nandurkar, H.H., Robb, L., Tarlinton, D., Barnett, L., Köntgen, F., and Begley, C.G. (1997). Adult mice with targeted mutation of the interleukin-11 receptor (IL11Ra) display normal hematopoiesis. Blood *90*, 2148–2159.

Neufert, C., Becker, C., and Neurath, M.F. (2007). An inducible mouse model of colon carcinogenesis for the analysis of sporadic and inflammation-driven tumor progression. Nat. Protoc. *2*, 1998–2004.

Oguma, K., Oshima, H., Aoki, M., Uchio, R., Naka, K., Nakamura, S., Hirao, A., Saya, H., Taketo, M.M., and Oshima, M. (2008). Activated macrophages promote Wnt signalling through tumour necrosis factor-alpha in gastric tumour cells. EMBO J. *27*, 1671–1681.

Onnis, B., Fer, N., Rapisarda, A., Perez, V.S., and Melillo, G. (2013). Autocrine production of IL-11 mediates tumorigenicity in hypoxic cancer cells. J. Clin. Invest. *123*, 1615–1629.

Orazi, A., Du, X., Yang, Z., Kashai, M., and Williams, D.A. (1996). Interleukin-11 prevents apoptosis and accelerates recovery of small intestinal mucosa in mice treated with combined chemotherapy and radiation. Lab. Invest. *75*, 33–42.

Popivanova, B.K., Kitamura, K., Wu, Y., Kondo, T., Kagaya, T., Kaneko, S., Oshima, M., Fujii, C., and Mukaida, N. (2008). Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis. J. Clin. Invest. *118*, 560–570.

Puchalski, T., Prabhakar, U., Jiao, Q., Berns, B., and Davis, H.M. (2010). Pharmacokinetic and pharmacodynamic modeling of an anti-interleukin-6 chimeric monoclonal antibody (siltuximab) in patients with metastatic renal cell carcinoma. Clin. Cancer Res. *16*, 1652–1661.

Quesnelle, K.M., Boehm, A.L., and Grandis, J.R. (2007). STAT-mediated EGFR signaling in cancer. J. Cell. Biochem. *102*, 311–319.

Rakoff-Nahoum, S., and Medzhitov, R. (2007). Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88. Science *317*, 124–127.

Rebouissou, S., Amessou, M., Couchy, G., Poussin, K., Imbeaud, S., Pilati, C., Izard, T., Balabaud, C., Bioulac-Sage, P., and Zucman-Rossi, J. (2009). Frequent in-frame somatic deletions activate gp130 in inflammatory hepatocellular tumours. Nature *457*, 200–204.

Rutter, M., Saunders, B., Wilkinson, K., Rumbles, S., Schofield, G., Kamm, M., Williams, C., Price, A., Talbot, I., and Forbes, A. (2004). Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis. Gastroenterology *126*, 451–459.

Santos, F.P., Kantarjian, H.M., Jain, N., Manshouri, T., Thomas, D.A., Garcia-Manero, G., Kennedy, D., Estrov, Z., Cortes, J., and Verstovsek, S. (2010). Phase 2 study of CEP-701, an orally available JAK2 inhibitor, in patients with primary or post-polycythemia vera/essential thrombocythemia myelofibrosis. Blood *115*, 1131–1136.

Schwitalla, S., Ziegler, P.K., Horst, D., Becker, V., Kerle, I., Begus-Nahrmann, Y., Lechel, A., Rudolph, K.L., Langer, R., Slotta-Huspenina, J., et al. (2013). Loss of p53 in enterocytes generates an inflammatory microenvironment enabling invasion and lymph node metastasis of carcinogen-induced colorectal tumors. Cancer Cell *23*, 93–106.

Sen, M., Joyce, S., Panahandeh, M., Li, C., Thomas, S.M., Maxwell, J., Wang, L., Gooding, W.E., Johnson, D.E., and Grandis, J.R. (2012). Targeting Stat3 abrogates EGFR inhibitor resistance in cancer. Clin. Cancer Res. *18*, 4986–4996.

Shin, S.Y., Choi, C., Lee, H.G., Lim, Y., and Lee, Y.H. (2012). Transcriptional regulation of the interleukin-11 gene by oncogenic Ras. Carcinogenesis *33*, 2467–2476.

Su, L.K., Kinzler, K.W., Vogelstein, B., Preisinger, A.C., Moser, A.R., Luongo, C., Gould, K.A., and Dove, W.F. (1992). Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science *256*, 668–670.

Tanaka, T., Suzuki, R., Kohno, H., Sugie, S., Takahashi, M., and Wakabayashi, K. (2005). Colonic adenocarcinomas rapidly induced by the combined treatment with 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and dextran sodium sulfate in male ICR mice possess beta-catenin gene mutations and increases immunoreactivity for beta-catenin, cyclooxygenase-2 and inducible nitric oxide synthase. Carcinogenesis *26*, 229–238.

Tebbutt, N.C., Giraud, A.S., Inglese, M., Jenkins, B., Waring, P., Clay, F.J., Malki, S., Alderman, B.M., Grail, D., Hollande, F., et al. (2002). Reciprocal regulation of gastrointestinal homeostasis by SHP2 and STAT-mediated trefoil gene activation in gp130 mutant mice. Nat. Med. *8*, 1089–1097.

Teramura, M., Kobayashi, S., Hoshino, S., Oshimi, K., and Mizoguchi, H. (1992). Interleukin-11 enhances human megakaryocytopoiesis in vitro. Blood *79*, 327–331.

Trainer, D.L., Kline, T., McCabe, F.L., Faucette, L.F., Feild, J., Chaikin, M., Anzano, M., Rieman, D., Hoffstein, S., Li, D.J., et al. (1988). Biological characterization and oncogene expression in human colorectal carcinoma cell lines. Int. J. Cancer *41*, 287–296.

Tye, H., Kennedy, C.L., Najdovska, M., McLeod, L., McCormack, W., Hughes, N., Dev, A., Sievert, W., Ooi, C.H., Ishikawa, T.O., et al. (2012). STAT3-driven upregulation of TLR2 promotes gastric tumorigenesis independent of tumor inflammation. Cancer Cell *22*, 466–478.

Wirtz, S., Neufert, C., Weigmann, B., and Neurath, M.F. (2007). Chemically induced mouse models of intestinal inflammation. Nat. Protoc. *2*, 541–546.

Yu, H., Kortylewski, M., and Pardoll, D. (2007). Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nat. Rev. Immunol. *7*, 41–51.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 17, pp. 13743–13751, April 20, 2012

Constitutively Active Mutant gp130 Receptor Protein from Inflammatory Hepatocellular Adenoma Is Inhibited by an Anti-gp130 Antibody That Specifically Neutralizes Interleukin 11 Signaling*

Received for publication, February 2, 2012 Published, JBC Papers in Press, March 6, 2012, DOI 10.1074/jbc.M111.349167

Jan Sommer[‡1], **Timo Effenberger**[§1], **Elena Volpi**[‡], **Georg H. Waetzig**[¶], **Marten Bernhardt**[§], **Jan Suthaus**[§], **Christoph Garbers**[‡], **Stefan Rose-John**[§], **Doreen M. Floss**[‡], **and Jürgen Scheller**[‡2]

From the [‡]*Institute of Biochemistry and Molecular Biology II, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany, the* [§]*Institute of Biochemistry, Medical Faculty, Christian-Albrechts-University, Kiel, Germany, and the* [¶]*CONARIS Research Institute AG, Kiel, Germany*

Background: Constitutively active, mutant gp130 is responsible for the development of inflammatory hepatocellular adenomas (IHCA).
Results: The anti-gp130 antibody B-P4 blocks constitutive activation of mutant gp130.
Conclusion: B-P4 might be a drug candidate for IHCAs and rare cases of gp130-associated hepatocellular carcinoma.
Significance: This is the first report on how to block oncogenic activation of gp130.

Ligand-independent constitutively active gp130 mutants were described to be responsible for the development of inflammatory hepatocellular adenomas (IHCAs). These variants had gain-of-function somatic mutations within the extracellular domain 2 (D2) of the gp130 receptor chain. Cytokine-dependent Ba/F3 cells were transduced with the constitutively active variant of gp130 featuring a deletion in the domain 2 from Tyr-186 to Tyr-190 (gp130ΔYY). These cells showed constitutive phosphorylation of signal transducer and activator of transcription-3 (STAT3) and cytokine-independent proliferation. Deletion of the Ig-like domain 1 (D1) of gp130, but not anti-gp130 mAbs directed against D1, abolished constitutive activation of gp130ΔYY, highlighting that this domain is involved in ligand-independent activation of gp130ΔYY. Moreover, soluble variants of gp130 were not able to inhibit the constitutive activation of gp130ΔYY. However, the inhibition of constitutive activation of gp130ΔYY was achieved by the anti-gp130 mAb B-P4, which specifically inhibits gp130 signaling by IL-11 but not by other IL-6 type cytokines. IL-11 but not IL-6 levels were found previously to be up-regulated in IHCAs, suggesting that mutations in gp130 are leading to IL-11-like signaling. The mAb B-P4 might be a valuable tool to inhibit the constitutive activation of naturally occurring gp130 mutants in IHCAs and rare cases of gp130-associated hepatocellular carcinoma.

Inflammatory hepatocellular adenoma (IHCA)[3] is a subtype of hepatocellular adenoma, which is a rare benign liver tumor mostly affecting younger females. IHCAs are characterized by polymorphic inflammatory cell infiltrates and activation of acute phase proteins, such as C-reactive protein and serum amyloid A (1). IHCAs show constitutive phosphorylation of signal transducer and activator of transcription 3 (STAT3), indicating a crucial role of gp130 signaling (2). The activation of gp130 receptor complexes leads to intracellular activation of Janus kinases (Jak/Tyk) as well as the STAT family of transcription factors such as STAT1 and STAT3. Furthermore, the activation leads to stimulation of the Ras/Raf/MAP kinase pathways (3). Importantly, the gp130 cytokine family member IL-6 is not overexpressed in IHCAs. However, about 60% of the investigated patient samples revealed small in-frame deletions within the binding site II of domain 2 (D2) of gp130 (2) and additional 12% carried activating STAT3 mutations (4). The marked activation of the gp130 signaling pathway in IHCAs was shown to be directly caused by these gain-of-function somatic mutations within the gp130 receptor chain, resulting in ligand-independent constitutively active mutant gp130 proteins (2). Sustained ligand-independent activation of gp130 homo- and heterotypic signaling pathways was demonstrated recently, showing that long-term activation was not suppressed by negative feedback loops (5, 6). Mutant gp130 receptor chains were coexpressed along with wild-type gp130, suggesting a dominant effect of the mutations. β-catenin mutations are frequently associated with IHCAs (2). The low transformation potential of IHCAs (below 5%) might be attributed to the coexistence of gp130-mutations plus activated β-catenin pathways (2).

The presented experiments show that gp130ΔYY also confers ligand-independent and sustained proliferation of Ba/F3-gp130ΔYY cells, which adds gp130 to the list of oncogenes. Moreover, the immunoglobulin-like domain 1 (D1) of gp130 is crucial for the receptor autoactivation of a frequent in-frame gp130 deletion variant spanning a deletion in the domain 2 (D2) from Tyr-186 to Tyr-190 and designated as gp130ΔYY. More-

* This work was funded by Deutsche Forschungsgemeinschaft (DFG, Bonn, Germany) SCHE 907/2-1, SFB877 A2, SFB841 C1.
[1] Both authors contributed equally to this work.
[2] To whom correspondence should be addressed: Institute of Biochemistry and Molecular Biology II, Medical Faculty, Heinrich-Heine-University, Universitätsstr. 1, 40225 Düsseldorf, Germany. Tel.: 49-211-8112724; Fax: 49-211-8112726; E-mail: jscheller@uni-duesseldorf.de.
[3] The abbreviations used are: IHCA, inflammatory hepatocellular adenoma; D1, domain 1; D2, domain 2; CBM, cytokine binding module.

over, we demonstrate the specific and efficient inhibition of autonomous gp130ΔYY receptor activation by the neutralizing anti-gp130 antibody B-P4, which specifically inhibits IL-11-mediated signaling.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Ba/F3 and Ba/F3-gp130 cells were obtained from Immunex (Seattle, WA) (7), COS-7 cells from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and Phoenix-Eco cells from U. Klingmüller (Deutsche Krebsforschungszentrum, Heidelberg, Germany). All cells were grown in DMEM high-glucose culture medium (PAA Laboratories, Cölbe, Germany) supplemented with 10% FBS, penicillin (60 mg/liter), and streptomycin (100 mg/liter) at 37 °C with 5% CO_2 in a water-saturated atmosphere. For cultivation of Ba/F3-gp130 cells, standard DMEM was supplemented with 10 ng/ml Hyper-IL-6. Hyper-IL-6 is a fusion protein of IL-6 and the soluble IL-6 receptor that mimics IL-6 trans-signaling (8, 9). Hyper-IL-6 was expressed and purified as described previously (8). Ba/F3 cells were cultured in the presence of IL-3 or conditioned medium from WEHI-3B cells, which constitutively produce IL-3. Anti-Myc-tag (71D10), anti-STAT1/3, and anti-phospho STAT1/3 mAbs were purchased from Cell Signaling Technology (Frankfurt am Main, Germany). Anti-c-myc (9E10) mAbs were from Santa Cruz Biotechnology (Heidelberg, Germany). The anti-gp130 mAb B-T2 was obtained from Abcam (Cambridge, UK), anti-gp130 mAb B-R3 from Santa Cruz Biotechnology, and anti-gp130 mAb B-P4 from Hölzel (Hölzel Diagnostika GmbH, Köln, Germany). The recombinant proteins sgp130 and sgp130Fc were expressed and purified as described previously (10).

Construction of Expression Plasmids—Standard cloning procedures were performed as described (11). The plasmid pBSK-gp130 was used as a template to amplify a fragment of gp130 coding for the deletion from Tyr-186 to Tyr-190 with the primer 5′gp130c2 (5′-GATATCGCCGCCATGTTGACGTT-GCAGACTTGGGT-3′) and 3′gp130delSY (5′-GACAAAAT-CAACAGTGCATGAGGTG-3′). The resulting PCR product was subcloned into pBSK-gp130 via HincII (vector) and EcoRV (insert) to obtain the plasmid pBSK-gp130ΔYY.

The deletion of the sequence coding for D1 of gp130 (from proline 27 to glycine 123) was performed by splicing by overlap-extension PCR, which preserved the original signal peptide coding sequence of gp130. The resulting plasmid was named pBSK-gp130ΔD1. For producing the deletion from Tyr-186 to Tyr-190 in the D2 domain of gp130, the plasmid pBSK-gp130ΔD1 was used as a template. Again, the resulting PCR-product was subcloned into pBSK-gp130ΔD1 via HincII (plasmid) and EcoRV (insert) to obtain pBSK-gp130ΔD1ΔYY.

The plasmid pEYFP-gp130 (12) was digested with NotI and EcoRI for obtaining the enhanced YFP (EYFP)-tagged C terminus of gp130, which was subcloned into pBSK-gp130ΔYY and pBSK-gp130ΔD1ΔYY. The resulting plasmids were named pBSK-gp130ΔYY-EYFP and pBSK-gp130ΔD1ΔYY-EYFP. These plasmids were used to create plasmids for the expression of C-terminal myc-tagged gp130 expression vectors by exchange of the EYFP with the myc tag (5′ primer 5′-GATCC-AGAACAAAAACTCATCTCAGAAGAGGATCTGTAGG-C-3′ and 3′ primer 5′-GGCCGCCTACAGATCCTCTTCTG-AGATGAGTTTTTGTTCTG-3′) using NotI and BamHI. The resulting plasmids were named pBSK-gp130ΔYY-myc and pBSK-gp130ΔD1ΔYY-myc.

The plasmid pBSK-gp130ΔYY was digested with HincII and ligated with an HincII fragment containing the gp130 signal peptide, myc tag, and a part of the gp130 extracellular domain (synthesized by GENEART, Regensburg, Germany), leading to pBSK-myc-gp130. To obtain the corresponding plasmid containing the deletion from Tyr-186 to Tyr-190, pBSK-myc-gp130 was used as a template to amplify a fragment of gp130 coding for the deletion with the primer 5′gp130c2 and 3′gp130delSY. The resulting plasmid was named pBSK-myc-gp130ΔYY. For deletion of D1, pBSK-myc-gp130 was digested with HincII, and a fragment coding for the signal peptide of gp130, a myc-tag, and a part of gp130 containing the deletion of D1 and Tyr-186 to Tyr-190 was inserted, resulting in pBSK-myc-gp130ΔD1ΔYY. cDNAs coding for gp130ΔYY, gp130ΔD1ΔYY, gp130ΔYY-myc, gp130ΔD1ΔYY-myc, myc-gp130, myc-gp130ΔYY, and myc-gp130ΔD1ΔYY were subcloned into the retroviral expression vector pMOWS (13). The resulting plasmids were named pMOWS-gp130ΔYY, pMOWS-gp130ΔD1ΔYY, pMOWS-gp130ΔYY-myc, pMOWS-gp130ΔD1ΔYY-myc, pMOWS-myc-gp130, pMOWS-myc-gp130ΔYY, and pMOWS-myc-gp130ΔD1ΔYY. The vector pMOWS-gp130-myc was obtained by digestion of pMOWS-gp130-EYFP and pMOWS-gp130ΔYY-myc with BlpI and HindIII. Subsequently, the resulting pMOWS vector backbone and the insert containing the C-terminal myc tag were ligated. cDNAs coding for myc-gp130, myc-gp130ΔYY, and myc-gp130ΔD1ΔYY were subcloned additionally into the expression plasmid p409 (14).

Transfection, Transduction, and Selection of Ba/F3-gp130 Cells—The murine pre-B cell line Ba/F3 and Ba/F3-gp130 cells, stably transduced with human gp130, were used for retroviral transduction with the plasmid derivatives of the retroviral expression vector pMOWS. For this purpose, pMOWS plasmids (1 μg each) were transiently transfected in 8×10^5 Phoenix-Eco cells using TurboFect™ according to manufacturer's instructions (Fermentas, St. Leon-Rot, Germany). The transfection efficiency was typically about 50%, which was estimated by GFP expression 24 h after transfection (Axiovert 200 microscope, Zeiss). Retroviral supernatants were produced as described (13). 250 μl of the retroviral supernatant were applied to 1×10^5 Ba/F3 or Ba/F3-gp130 cells and mixed, and the solution was centrifuged at 1800 rpm for 2 h at 21 °C in the presence of polybrene (8 μg/ml). Transduced cells were grown in standard medium supplemented with either 10 ng/ml IL-3 (Ba/F3 cells) or 10 ng/ml Hyper-IL-6 (Ba/F3-gp130 cells). 48 h after transduction, transduced cells were selected in 1.5 μg/ml puromycin (PAA Laboratories) for at least 2 weeks. After 2 weeks of antibiotic selection in the presence of IL-3 or Hyper-IL-6, the cells were screened for cytokine-independent proliferation.

Proliferation Assays—Transduced Ba/F3-gp130 cells expressing the gp130 variants with or without the myc tag were washed three times with sterile PBS and suspended in DMEM containing 10% FBS at 5×10^3 cells per well of a 96-well plate. The cells were cultured for 3 days in a final volume of 100 μl

with or without additional cytokines or antibodies as indicated. The CellTiter-Blue® cell viability assay (Promega, Mannheim, Germany) was used to determine the cell number following the manufacturer's instructions and measured on a Lambda Fluoro 320 fluorometer (excitation filter 530/25, emission filter 590/35, sensitivity 75, software KC4). Relative light unit values were normalized by subtractions of negative control values (unstimulated Ba/F3-gp130 cells) from all other values. All values were measured in triplicates.

Western Blotting—For detection of phospho-STAT3, cells were washed three times with sterile PBS and starved for 6 h in serum-free DMEM before adding additional cytokines or sgp130Fc as indicated. Subsequently, cells were centrifuged, and the pellet was directly frozen in liquid nitrogen. Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 1% IGEPAL (Nonidet P-40) and 1% Trition-X-100, supplemented with complete protease inhibitor mixture tablets (Roche)).

Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (GE Healthcare). The membrane was blocked with 5% skimmed milk in Tris-buffered saline with Tween 20 (TBS-T; 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.5% Tween 20) and probed with primary antibodies as indicated at 4 °C overnight. After washing with TBS-T, the membranes were incubated with the appropriate secondary antibodies conjugated to HRP (Thermo Scientific/Pierce, Perbio), and protein bands were visualized with the ECL detection system (GE Healthcare) according to the manufacturer's instructions.

Flow Cytometry Staining and Analysis—To detect the surface expression of N-terminally myc-tagged gp130 variants, cells were washed with FACS buffer (PBS, 1% BSA) and incubated at 5×10^5 cells/100 μl of FACS buffer containing 1:100 diluted anti-myc tag (71D10) mAb (Cell Signaling Technology) in FACS buffer for 60 min on ice. After a single washing step in FACS buffer, cells were incubated in 100 μl of FACS buffer containing a 1:100 dilution of Alexa Fluor 488-conjugated anti-rabbit mAb (Life Technology, Darmstadt, Germany), respectively. Cells were washed once with FACS buffer, resuspended, and analyzed by flow cytometry (BD Biosciences, FACSCantoII and FACS DIVA software). Detection of gp130 on the cell surface was further performed with mouse anti-gp130 (B-R3) mAb (sc-57189, Santa Cruz Biotechnology) followed by allophycocyanin-conjugated AffiniPure $F(ab')_2$ fragment goat anti-mouse IgG (Dianova, Hamburg, Germany).

Coprecipitation Studies Using the Nanotrap System—For coprecipitation, transiently transfected COS-7 cells were collected by scraping and subsequently lysed in 200 μl of lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 2 mM PMSF, 0.5% Nonidet P-40). The volume of the lysate was adjusted to 500 μl with dilution buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 2 mM PMSF). 50 μl of each lysate was boiled with 50 μl 4× Laemmli buffer. The remaining 450 μl of lysate was incubated with anti-GFP-specific nanobodies (25 μg), coupled to N-hydroxysuccinimide-activated Sepharose as described previously (15). The mixture was incubated at room temperature in an overhead rotator for 2 h. Afterward, the Sepharose was washed four times with 250 μl dilution buffer and subsequently boiled in 100 μl 2x Laemmli buffer. The lysate and the precipitated proteins were separated by SDS-PAGE and analyzed by Western blotting using mAbs against the myc-tag or GFP and the appropriate secondary antibodies conjugated to horseradish peroxidase (Thermo Scientific). Protein bands were visualized with the ECL detection system (GE Healthcare) according to the manufacturer's instructions.

RESULTS

Cell-autonomous Proliferation of Ba/F3 Cells by the Ligand-independent, Constitutively Active gp130 Variant gp130ΔYY—We have generated the ligand-independent constitutively active gp130 variant gp130ΔYY, featuring a deletion in the domain 2 (D2) from Tyr-186 to Tyr-190 (Fig. 1*A*). This variant was selected because it represents four of 26 identified mutations in IHCAs (2). Moreover, 20 of 26 patients carried deletions that include this region or a deletion from ΔSer-187 to Tyr-190 (gp130ΔSY, six of 26). Therefore, we conclude that amino acids Tyr-186 to Tyr-190 of gp130 are representative for most of the ligand-independent gp130 receptor variants. The murine pre-B cell line Ba/F3 was chosen as a model system to investigate the constitutive activation of gp130ΔYY. Ba/F3 cells usually grow in dependence of the cytokine IL-3. However, after transduction with the gp130 receptor chain cDNA, Ba/F3-gp130 cells grow in the presence of IL-6 and the soluble IL-6R or Hyper-IL-6, which is a fusion protein thereof (8, 9). The cDNA encoding gp130ΔYY was stably transduced into Ba/F3-gp130 cells (Ba/F3-gp130-gp130ΔYY) because Ba/F-3 cells expressing the wild-type and the mutated gp130 receptor reflected the *in vivo* situation, with heterozygous cells having a wild-type and a mutated gp130 allele. Ba/F3-gp130-gp130ΔYY cells showed ligand-independent STAT3 phosphorylation and long-term proliferation, indicating that gp130ΔYY confers a dominant ligand-independent, cell-autonomous gp130 receptor activation phenotype (Fig. 1, *B* and *C*). Even though STAT3 phosphorylation has already been shown for transiently transfected Hep3B cells (2), it remained elusive whether gp130ΔYY also mediates long-term receptor activation and cellular proliferation.

Because Ba/F3-gp130-gp130ΔYY cells also expressed the wild-type gp130 receptor, it was not possible to prove protein expression of the untagged gp130ΔYY protein in these cells. The size difference between wild-type gp130 and gp130ΔYY was only five amino acids, and both gp130 receptor variants were almost undetectable by Western blotting using anti-gp130 antibodies (data not shown). Therefore, stably transduced Ba/F3-gp130 cells with C-terminally myc-tagged wild-type gp130 and gp130ΔYY proteins (referred to as Ba/F3-gp130-gp130-myc and Ba/F3-gp130-gp130ΔYY-myc) were generated. Expression of the corresponding cDNAs was demonstrated by Western blotting with anti-myc mAbs (Fig. 1*D*). Again, only gp130ΔYY-myc transduced Ba/F3-gp130-cells showed cytokine-independent proliferation and STAT3 phosphorylation (Fig. 1, *E* and *F*).

Interestingly, the wild-type gp130 receptor present in Ba/F3-gp130-gp130ΔYY cells did not interfere with cytokine-independent proliferation and STAT3 activation induced by

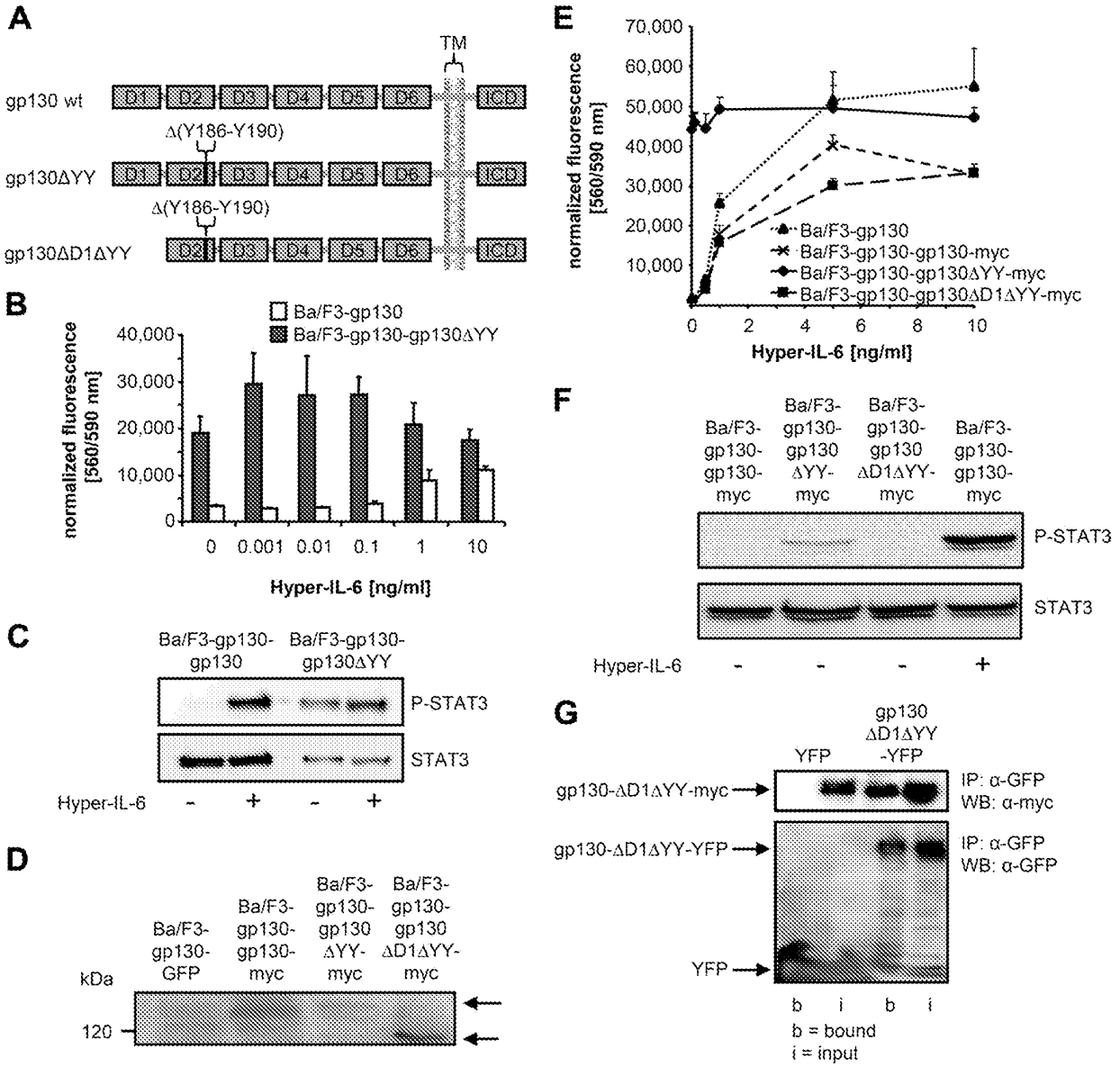


FIGURE 1. **Biological activity of gp130ΔYY and gp130ΔD1ΔYY in stably transduced Ba/F3-gp130-gp130ΔYY and Ba/F3-gp130-gp130ΔD1ΔYY cells.** *A*, schematic illustration of wild-type, gp130ΔYY, and gp130ΔD1ΔYY. *B*, equal numbers of Ba/F3-gp130 cells stably transduced with gp130ΔYY were cultured for 3 days in the presence or absence of Hyper-IL-6. Proliferation was measured as indicated under "Experimental Procedures." *TM*, transmembrane domain; *ICD*, intracellular domain. *C*, after 6 h of serum starvation, Ba/F3-gp130 cells stably transduced with gp130ΔYY were treated with Hyper-IL-6 for 5 min or left untreated. Phosphorylated STAT3 was detected by Western blot analysis. *D*, Western blot detection of C-terminal myc-tagged gp130ΔYY, gp130ΔD1ΔYY, and gp130. GFP served as a negative control. *Arrows* indicate the respective bands for gp130/gp130ΔYY and gp130ΔD1ΔYY. *E*, equal numbers of Ba/F3-gp130 cells stably transduced with gp130ΔYY-myc, gp130ΔD1ΔYY-myc, or gp130-myc were cultured for 3 days in the presence or absence of Hyper-IL-6. Proliferation was measured as indicated under "Experimental Procedures." *F*, after 6 h of serum starvation, Ba/F3-gp130 cells stably transduced with gp130-myc, gp130ΔYY-myc, or gp130ΔD1ΔYY-myc were analyzed for STAT3 phosphorylation. The membrane was stripped and reprobed with anti-STAT3. As a control, Ba/F3-gp130-gp130-myc cells were stimulated for 5 min with Hyper-IL-6 or left untreated. *G*, coprecipitation of gp130ΔD1ΔYY-myc with gp130ΔD1ΔYY-EYFP using the Nanotrap anti-GFP system as described under "Experimental Procedures. *IP*, immunoprecipitation; *WB*, Western blot.

gp130ΔYY. Here, we cannot exclude that the expression of wild-type gp130 receptor was too low to observe inhibition of gp130ΔYY. Inhibition of gp130ΔYY was demonstrated for Hep3B cells overexpressing both the wild-type and mutant gp130 receptor, albeit with an excess of the wild-type receptor (2). Of note, Ba/F3-gp130-gp130ΔYY-myc cells were selected for cytokine-independent growth, thus preferentially selecting clones that express a wild-type gp130/gp130ΔYY ratio that promotes gp130ΔYY activation.

Cell-autonomous Proliferation of Ba/F3 Cells by the Ligand-independent, Constitutively Active gp130 Variant gp130ΔYY Is Dependent on the D1 Domain of gp130—The extracellular part of the gp130 receptor consists of one immunoglobulin-like domain (Ig-like, D1), the cytokine binding module (CBM, D2

and D3), and three fibronectin-like type III domains (FNIII, D4-D6) (16). The binding of IL-6 and IL-11 to gp130 is mediated by the D1 domain of gp130 via site III of IL-6/IL-11 and the CBM of gp130 via site II of IL-6/IL-11 (17). A gp130 receptor lacking the Ig-like domain 1 (gp130ΔD1) is functionally inactive because gp130ΔD1 cannot form homodimers because of the missing binding site III for IL-6/IL-11 (18). To answer the question whether the D1 domain is crucial for the biological activity of gp130ΔYY, we genetically deleted the D1 domain from gp130ΔYY-myc (named gp130ΔD1ΔYY-myc) and generated Ba/F3-gp130-gp130ΔD1ΔYY-myc cells (Fig. 1*A*). As depicted in Fig. 1*D*, the gp130ΔD1ΔYY-myc protein was produced by Ba/F3-gp130-gp130ΔD1ΔYY-myc cells. However, Ba/F3-gp130-gp130ΔD1ΔYY-myc cells were not able to proliferate cytokine-independently and showed no constitutive STAT3 phosphorylation (Fig. 1, *E* and *F*). Published coprecipitation studies showed that constitutively active gp130ΔSY interacts with gp130ΔSY and wild-type gp130 in the absence of a ligand (2). Surprisingly, the biologically inactive variant gp130ΔD1ΔYY was also precipitated with gp130ΔD1ΔYY in the absence of any ligand (Fig. 1*G*), indicating that gp130ΔD1ΔYY can form inactive homodimers. These results suggested that the D1 domain of gp130ΔYY is crucial for ligand-independent receptor activation.

Using C-terminally tagged variants of gp130, we were not able to show that the gp130ΔD1ΔYY-myc variant was transported to the cell surface. Therefore, N-terminally tagged gp130 variants (myc-gp130, myc-gp130ΔYY, and myc-gp130ΔD1ΔYY) were generated and stably transduced into Ba/F3 and Ba/F3-gp130 cells. We were, however, not able to detect the N-terminal myc-tagged gp130 variants in lysates of Ba/F3 cells by Western blot analysis, but expression was verified in COS-7 cells (Fig. 2*A*). As expected, only the N-terminally tagged gp130ΔYY conferred ligand-independent proliferation of Ba/F3-gp130-myc-gp130ΔYY cells (Fig. 2*B*). Moreover, naive Ba/F3 cells stably transduced with myc-gp130, myc-gp130ΔYY, or myc-gp130ΔD1ΔYY were generated and tested for STAT3 phosphorylation with and without Hyper-IL-6 stimulation. Ba/F3-myc-gp130 showed Hyper-IL-6-induced STAT3 phosphorylation, Ba/F3-myc-gp130ΔYY showed STAT3 phosphorylation in the absence of cytokine stimulation, and Ba/F3-myc-gp130ΔD1ΔYY showed no STAT3 phosphorylation irrespective of cytokine stimulation (Fig. 2*C*). Even though Ba/F3-myc-gp130 cells showed STAT3 phosphorylation after Hyper-IL-6 stimulation, these cells did not shift from IL-3-dependent to Hyper-IL-6-dependent proliferation. However, after long-term cultivation with IL-3, Ba/F3-myc-gp130ΔYY cells converted to ligand-independent proliferation. For unknown reasons, we were not able to adopt the proliferation of Ba/F3-gp130 cells that were freshly transduced with wild-type gp130, to Hyper-IL-6-dependent growth (Fig. 2*D*). Finally, cell surface expression of all N-terminally myc-tagged gp130 variants of stably transduced Ba/F3 and Ba/F3-gp130 cells was shown by FACS analysis using an anti-myc mAb, excluding the possibility that the biological inactivity of gp130ΔD1ΔYY was due to inefficient transport to the plasma membrane (Fig. 2*E*). Furthermore, cell surface expression of gp130 variants of generated Ba/F3 cells was confirmed using the anti-gp130 mAb B-R3 (Fig. 2*E*).

The Constitutively Active gp130 Variant gp130ΔYY Is Inhibited by the Anti-gp130 Antibody B-P4—The D1 domain was needed for ligand-independent activation of gp130ΔYY. Therefore, we hypothesized that the mAb B-T2, which binds to the D1 domain and inhibits IL-6-induced activation of gp130, might also inhibit gp130ΔYY-induced proliferation (19). As expected, B-T2 inhibited Hyper-IL-6 induced proliferation of Ba/F3-gp130-myc-gp130 cells in a dose-dependent manner, but the proliferation of Ba/F3-gp130-myc-gp130ΔYY cells was not inhibited (Fig. 3*A*). From this experiment, we concluded that even though the D1 domain of gp130 was critical for cytokine-independent proliferation mediated by gp130ΔYY, the binding of IL-6 via site III to gp130 was not involved in the constitutive activation of gp130ΔYY.

Because we did not observe an inhibitory effect of wild-type gp130 on gp130ΔYY-induced cell proliferation and STAT3 phosphorylation in Ba/F3-gp130-gp130ΔYY cells, we tested whether the soluble gp130 (sgp130) or the fusion protein sgp130Fc, which both contain all extracellular domains of gp130, inhibit the ligand-independent activation of gp130ΔYY. Sgp130 is thought to be the natural inhibitor of IL-6 trans-signaling via the soluble IL-6·IL-6 receptor complex (10). Hyper-IL-6-induced proliferation of Ba/F3-gp130-gp130-myc cells was inhibited in a dose-dependent manner by sgp130 and sgp130Fc as described previously (Fig. 3, *B* and *C*) (10). However, sgp130 and dimeric sgp130Fc did not inhibit proliferation of Ba/F3-gp130-gp130ΔYY-myc cells. Furthermore, activation of STAT3 was efficiently inhibited by sgp130Fc in Ba/F3-myc-gp130 cells but not in the corresponding myc-gp130ΔYY cells (Fig. 3*D*).

Next, we tested two other neutralizing mAbs against gp130 for inhibition of the ligand-independent activation of gp130ΔYY. The mAb B-R3 is directed against the CBM (domain 2 of gp130) (19, 20), whereas the mAb B-P4 binds to the first of three fibronectin domains (domain 4 of gp130) (19, 21). As shown in Fig. 4*A*, B-R3 inhibited the proliferation of Ba/F3-gp130 cells stimulated with Hyper-IL-6 in a dose-dependent manner. However, the proliferation of Ba/F3-gp130-gp130ΔYY or Ba/F3-gp130-L-gp130 cells was not affected by B-R3. In L-gp130, the entire extracellular portion of gp130 was replaced with the c-jun leucine zipper region (5). As a control, B-R3 did not inhibit the proliferation of Ba/F3-gp130 cells stimulated with IL-3, indicating that B-R3 specifically blocked the receptor activation of gp130 in Ba/F3-gp130 cells (Fig. 4*B*). The binding epitope of B-R3 is within the CBM (D2). The failure of B-R3 to inhibit gp130ΔYY-induced cellular proliferation cannot be caused by the inability of B-R3 to bind to gp130ΔYY because this mAb was successfully used for detection of gp130, gp130ΔYY, and gp130ΔD1ΔYY in flow cytometry (Fig. 2*E*).

Interestingly, B-P4 specifically inhibited the proliferation of Ba/F3-gp130-gp130ΔYY in a concentration-dependent manner. Proliferation of Ba/F3-gp130-L-gp130 cells and Hyper-IL-6-induced proliferation of Ba/F3-gp130 cells was not inhibited (Fig. 4*C*). It was described previously that B-P4 specifically inhibits gp130 receptor activation exclusively induced by IL-11 but not by IL-6 (Hyper-IL-6), leukemia inhibitory factor,

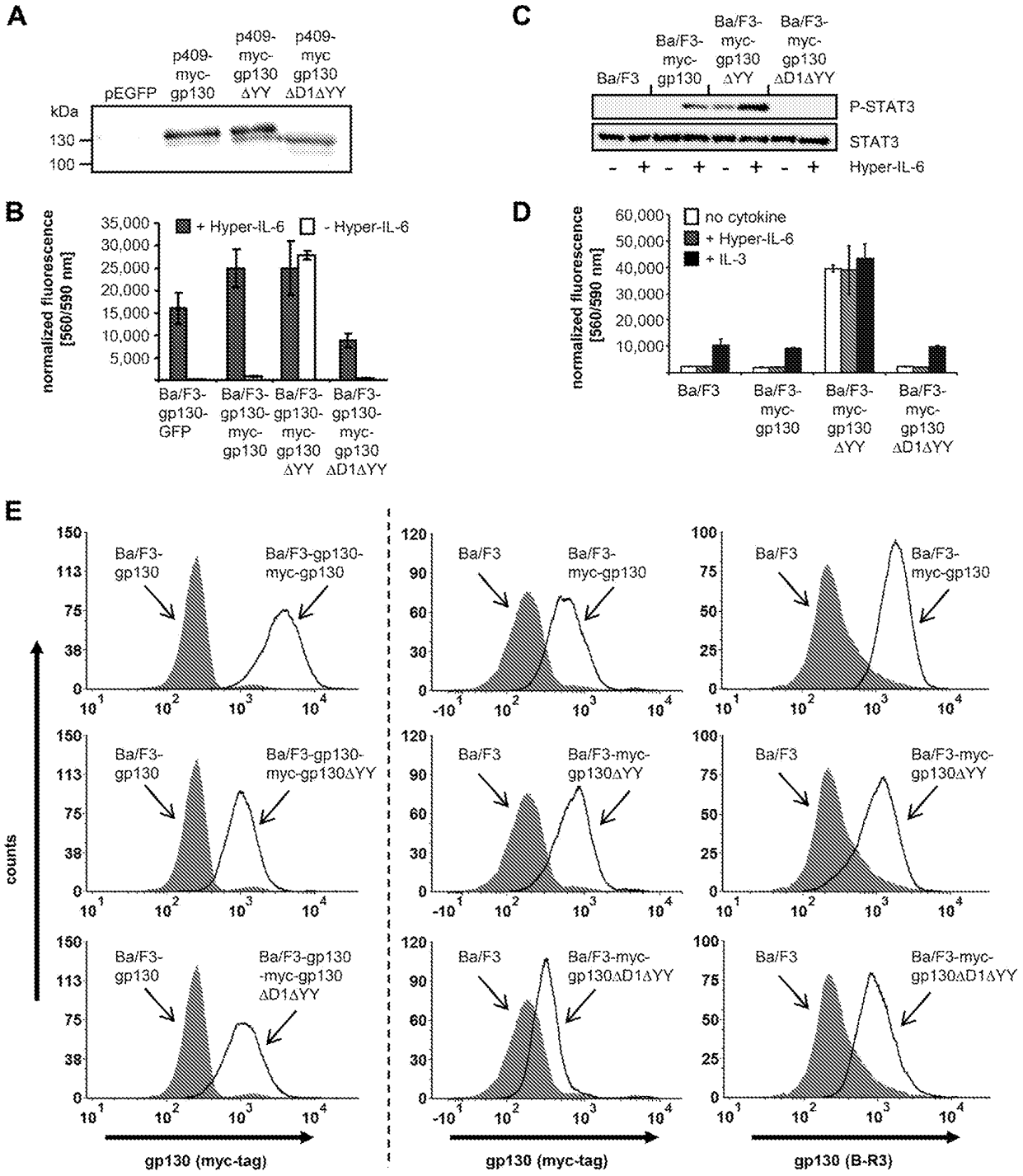


FIGURE 2. **Gp130ΔYY and gp130ΔD1ΔYY are presented on the cell surface of stably transduced Ba/F3 cells.** *A*, Western blot detection of myc-tagged gp130ΔYY, gp130ΔD1ΔYY, and gp130 in transiently transfected COS-7 cells using anti-myc-mAbs. GFP-transfected cells served as a negative control. *B*, equal numbers of Ba/F3-gp130 cells stably transduced with myc-gp130ΔYY, myc-gp130ΔD1ΔYY, myc-gp130, or GFP were cultured for 3 days in the presence or absence of Hyper-IL-6. Proliferation was measured as indicated under "Experimental Procedures." *C*, after 6 h of serum starvation, Ba/F3 cells stably transduced with myc-gp130ΔYY, myc-gp130ΔD1ΔYY, or myc-gp130 were stimulated for 5 min with Hyper-IL-6 or left untreated. Cells were analyzed for STAT3 phosphorylation by Western blotting. Membranes were stripped and reprobed with anti-STAT3 mAbs. As a negative control, Ba/F3 cells were used. *D*, equal numbers of Ba/F3 cells stably transduced with myc-gp130ΔYY, myc-gp130ΔD1ΔYY, or myc-gp130 were cultured for 3 days in the presence or absence of IL-3 or Hyper-IL-6. Proliferation was measured as indicated under "Experimental Procedures." *E*, cell surface expression of N-terminally myc-tagged gp130, gp130ΔYY, or gp130ΔD1ΔYY (*open histograms*) in stably transduced Ba/F3-gp130 and Ba/F3 cells was analyzed by FACS. Ba/F3-gp130 (*left panel*) and Ba/F3 (*center* and *right panels*) cells were used as negative controls (*filled histograms*).