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Application Number: 14462441 Document Date: 08/18/2014

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Form Revision Date: August 26, 2013

PTO/AIA/15 (03-13)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	86399-007740US-913296
First Named Inventor	Yaworski
Title	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
Express Mail Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. [ ] **Fee Transmittal Form** (PTO/SB/17 or equivalent)
2. [ ] **Applicant asserts small entity status.** See 37 CFR 1.27
3. [ ] **Applicant certifies micro entity status.** See 37 CFR 1.29. Applicant must attach form PTO/SB/15A or B or equivalent.
4. [✓] **Specification** [*Total Pages* 119] Both the claims and abstract must start on a new page. *(See MPEP § 608.01(a) for information on the preferred arrangement)*
5. [✓] **Drawing(s)** (35 U.S.C. 113) [*Total Sheets* 24]
6. **Inventor's Oath or Declaration** [*Total Pages* 5] *(including substitute statements under 37 CFR 1.64 and assignments serving as an oath or declaration under 37 CFR 1.63(e))*
   a. [ ] Newly executed (original or copy)
   b. [✓] A copy from a prior application (37 CFR 1.63(d))
7. [✓] **Application Data Sheet** ** See note below.* See 37 CFR 1.76 (PTO/AIA/14 or equivalent)
8. **CD-ROM or CD-R** in duplicate, large table, or Computer Program (*Appendix*)
   [ ] Landscape Table on CD
9. **Nucleotide and/or Amino Acid Sequence Submission** (*if applicable, items a. – c. are required*)
   a. [✓] Computer Readable Form (CRF)
   b. [ ] Specification Sequence Listing on:
      i. [ ] CD-ROM or CD-R (2 copies); or
      ii. [ ] Paper
   c. [ ] Statements verifying identity of above copies

ADDRESS TO: **Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450**

ACCOMPANYING APPLICATION PAPERS

10. [ ] **Assignment Papers** (cover sheet & document(s)) Name of Assignee _____
11. [✓] **37 CFR 3.73(c) Statement** (*when there is an assignee*) [✓] **Power of Attorney**
12. [ ] **English Translation Document** (*if applicable*)
13. [ ] **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    [ ] Copies of citations attached
14. [ ] **Preliminary Amendment**
15. [ ] **Return Receipt Postcard** (*MPEP § 503*) (*Should be specifically itemized*)
16. [ ] **Certified Copy of Priority Document(s)** (*if foreign priority is claimed*)
17. [ ] **Nonpublication Request** Under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
18. [ ] **Other:** _____

***Note:** (1) Benefit claims under 37 CFR 1.78 and foreign priority claims under 1.55 **must** be included in an Application Data Sheet (ADS).
(2) For applications filed under 35 U.S.C. 111, the application must contain an ADS specifying the applicant if the applicant is an assignee, person to whom the inventor is under an obligation to assign, or person who otherwise shows sufficient proprietary interest in the matter. See 37 CFR 1.46(b).

19. CORRESPONDENCE ADDRESS

[✓] The address associated with Customer Number: 20350 **OR** [ ] Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone		Email	

Signature	Joe C. Hao	Date	August 18, 2014
Name (Print/Type)	Joe C. Hao	Registration No. (Attorney/Agent)	55246

PTO/AIA/01 (06-12)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number 13/928,309

filed on June 26, 2013.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: KIEU LAM Date (Optional): 23-Oct-2013

Signature: [signature]

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

PTO/AIA/01 (06-12)
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LEGAL NAME OF INVENTOR

Inventor: LORNE PALMER Date (Optional): Oct 18/13

Signature: Lorne Palmer

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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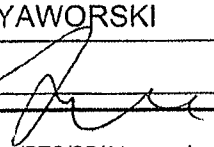
LEGAL NAME OF INVENTOR

Inventor: EDWARD YAWORSKI Date (Optional): Oct 18/13

Signature: 

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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LEGAL NAME OF INVENTOR

Inventor: LLOYD JEFFS Date (Optional): 18-OCT-2013

Signature: _____

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

PTO/AIA/01 (06-12)
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☒ United States application or PCT international application number 13/928,309 filed on June 26, 2013.

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LEGAL NAME OF INVENTOR

Inventor: IAN MacLACHLAN Date (Optional): October 28, 2013

Signature: _____

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

PTO/AIA/14 (03-13)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	86399-007740US-913296
	Application Number	
Title of Invention	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Remove

Inventor 1

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Edward		Yaworski	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Maple Ridge	Country of Residence [i]	CA

Mailing Address of Inventor:

Address 1	23106-123B Avenue		
Address 2			
City	Maple Ridge	**State/Province**	BC
Postal Code	V2X9Z7	**Country** [i]	CA

Remove

Inventor 2

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Kieu		Lam	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Surrey	Country of Residence [i]	CA

Mailing Address of Inventor:

Address 1	18871 71 Avenue		
Address 2			
City	Surrey	**State/Province**	BC
Postal Code	V4N5M7	**Country** [i]	CA

Remove

Inventor 3

Legal Name

PTO/AIA/14 (03-13)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	86399-007740US-913296
	Application Number	
Title of Invention	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY	

Prefix	Given Name	Middle Name	Family Name	Suffix
	Lloyd		Jeffs	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Delta	Country of Residence [i]	CA

Mailing Address of Inventor:

Address 1	5317 Laurel Drive		
Address 2			
City	Delta	State/Province	BC
Postal Code	V4K3S4	Country [i]	CA

Remove

Inventor 4

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Lorne		Palmer	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Vancouver	Country of Residence [i]	CA

Mailing Address of Inventor:

Address 1	8076 Elliott Street		
Address 2			
City	Vancouver	State/Province	BC
Postal Code	V5S2P2	Country [i]	CA

Remove

Inventor 5

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Ian		MacLachlan	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Mission	Country of Residence [i]	CA

Mailing Address of Inventor:

Address 1	8040 Aves Terrace		
Address 2			
City	Mission	State/Province	BC
Postal Code	V4S1E5	Country [i]	CA

PTO/AIA/14 (03-13)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	86399-007740US-913296
	Application Number	
Title of Invention	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY	

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below. For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	20350		
Email Address	ipefiling@kilpatricktownsend.com	Add Email	Remove Email

Application Information:

Title of the Invention	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY		
Attorney Docket Number	86399-007740US-913296	**Small Entity Status Claimed** ☐	
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	24	**Suggested Figure for Publication (if any)**	

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32). Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	20350		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.

Prior Application Status	Pending	Remove

PTO/AIA/14 (03-13)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	86399-007740US-913296
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Title of Invention	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY	

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
	Continuation of	13/928309	2013-06-26
Prior Application Status	Patented		Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
13/928309	Continuation of	13/253917	2011-10-05	8492359	2013-07-23
Prior Application Status	Patented				Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
13/253917	Continuation of	12/424367	2009-04-15	8058069	2011-11-15
Prior Application Status	Expired				Remove

Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
12/424367	non provisional of	61/045228	2008-04-15

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(d). When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(h)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Remove

Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

PTO/AIA/14 (03-13)
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	Application Number	
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Authorization to Permit Access:

☒ Authorization to Permit Access to the Instant Application by the Participating Offices

If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the instant patent application is filed access to the instant patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the instant patent application is filed to have access to the instant patent application.

In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the instant patent application with respect to: 1) the instant patent application-as-filed; 2) any foreign application to which the instant patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the instant patent application; and 3) any U.S. application-as-filed from which benefit is sought in the instant patent application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date o f filing this Authorization.

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

◉ Assignee	○ Legal Representative under 35 U.S.C. 117	○ Joint Inventor
○ Person to whom the inventor is obligated to assign.	○ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor :

If the Applicant is an Organization check here. ☒

Organization Name	Protiva Biotherapeutics, Inc.

PTO/AIA/14 (03-13)
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Mailing Address Information For Applicant:			
Address 1	100-8900 Glenlyon Parkway		
Address 2			
City	Burnaby	**State/Province**	BC
Country i	CA	Postal Code	V5J5J8
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button.

Non-Applicant Assignee Information:

Providing assignment information in this section does not subsitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Assignee 1

Complete this section only if non-applicant assignee information is desired to be included on the patent application publication in accordance with 37 CFR 1.215(b). Do not include in this section an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest), as the patent application publication will include the name of the applicant(s).

If the Assignee is an Organization check here. ☐

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	86399-007740US-913296
	Application Number	
Title of Invention	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY	

Signature:

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Signature				Date (YYYY-MM-DD)	2014-08-18
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I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(c).

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Assignee Name and Address: PROTIVA BIOTHERAPEUTICS, INC.
100-8900 Glenlyon Parkway, Burnaby, BC V5J-5J8

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SIGNATURE of Assignee of Record

The individual whose signature and title is supplied below is authorized to act on behalf of the assignee

Signature	Barry McGurl	Date	March 5 2014.
Name	Barry McGurl	Telephone	604 419-3230
Title	Director, IP		

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STATEMENT UNDER 37 CFR 3.73(b)

Applicant/Patent Owner: Protiva Biotherapeutics, Inc.

Application No./Patent No.: TBD- filed herewith Filed/Issue Date: 08/18/2014

Titled: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

PROTIVA BIOTHERAPEUTICS, INC. , a corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.

states that it is:

1. ☒ the assignee of the entire right, title, and interest in;

2. ☐ an assignee of less than the entire right, title, and interest in (The extent (by percentage) of its ownership interest is _____ %); or

3. ☐ the assignee of an undivided interest in the entirety of (a complete assignment from one of the joint inventors was made)

the patent application/patent identified above, by virtue of either:

A. ☐ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy therefore is attached.

OR

B. ☒ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: MacLachlan, Yaworski, & Lam To: Protiva Biotherapeutics, Inc.

The document was recorded in the United States Patent and Trademark Office at
Reel 023628 , Frame 0829 , or for which a copy thereof is attached.

2. From: Jeffs & Palmer To: Protiva Biotherapeutics, Inc.

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☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☒ As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (*i.e.*, a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

Joe C. Hao — August 18, 2014
Signature — Date

Joe C. Hao — Attorney for Applicant/Assignee
Printed or Typed Name — Title

NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] The present application is a continuation of U.S. Application No. 13/928,309, filed June 26, 2013, which application is a continuation of 13/253,917, filed October 5, 2011, now U.S. Patent No. 8,492,359, which application is a continuation of 12/424,367 filed April 15, 2009, now U.S. Patent No. 8,058,069, which application claims priority to U.S. Provisional Application No. 61/045,228, filed April 15, 2008, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] Not applicable.

NAMES OF PARTIES TO A JOINT RESEARCH AGREEMENT

[0003] Not applicable.

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED AS AN ASCII TEXT FILE

[0004] The Sequence Listing written in file -77-3.TXT, created on August 22, 2013, 8,192 bytes, machine format IBM-PC, MS-Windows operating system, is hereby incorporated by reference in its entirety for all purposes.

BACKGROUND OF THE INVENTION

[0005] RNA interference (RNAi) is an evolutionarily conserved process in which recognition of double-stranded RNA (dsRNA) ultimately leads to posttranscriptional suppression of gene expression. This suppression is mediated by short dsRNA, also called small interfering RNA (siRNA), which induces specific degradation of mRNA through complementary base pairing. In several model systems, this natural response has been developed into a powerful tool for the investigation of gene function (*see, e.g.,* Elbashir *et al.*, *Genes Dev.*, 15:188-200 (2001); Hammond *et al.*, *Nat. Rev. Genet.*, 2:110-119 (2001)). More recently, it was discovered that introducing synthetic 21-nucleotide dsRNA duplexes into mammalian cells could efficiently silence gene expression.

[0006] Although the precise mechanism is still unclear, RNAi provides a potential new approach to downregulate or silence the transcription and translation of a gene of interest. For example, it is desirable to modulate (*e.g.*, reduce) the expression of certain genes for the treatment of neoplastic disorders such as cancer. It is also desirable to silence the expression of genes associated with liver diseases and disorders such as hepatitis. It is further desirable to reduce the expression of certain genes for the treatment of atherosclerosis and its manifestations, *e.g.,* hypercholesterolemia, myocardial infarction, and thrombosis.

[0007] A safe and effective nucleic acid delivery system is required for RNAi to be therapeutically useful. Viral vectors are relatively efficient gene delivery systems, but suffer from a variety of limitations, such as the potential for reversion to the wild-type as well as immune response concerns. As a result, nonviral gene delivery systems are receiving increasing attention (Worgall *et al.*, *Human Gene Therapy*, 8:37 (1997); Peeters *et al.*, *Human Gene Therapy*, 7:1693 (1996); Yei *et al.*, *Gene Therapy*, 1:192 (1994); Hope *et al.*, *Molecular Membrane Biology*, 15:1 (1998)). Furthermore, viral systems are rapidly cleared from the circulation, limiting transfection to "first-pass" organs such as the lungs, liver, and spleen. In addition, these systems induce immune responses that compromise delivery with subsequent injections.

[0008] Plasmid DNA-cationic liposome complexes are currently the most commonly employed nonviral gene delivery vehicles (Felgner, *Scientific American*, 276:102 (1997); Chonn *et al.*, *Current Opinion in Biotechnology*, 6:698 (1995)). For instance, cationic liposome complexes made of an amphipathic compound, a neutral lipid, and a detergent for transfecting insect cells are disclosed in U.S. Patent No. 6,458,382. Cationic liposome complexes are also disclosed in U.S. Patent Publication No. 20030073640.

[0009] Cationic liposome complexes are large, poorly defined systems that are not suited for systemic applications and can elicit considerable toxic side effects (Harrison *et al.*, *Biotechniques*, 19:816 (1995); Li *et al.*, *The Gene*, 4:891 (1997); Tam *et al*, *Gene Ther.*, 7:1867 (2000)). As large, positively charged aggregates, lipoplexes are rapidly cleared when administered *in vivo*, with highest expression levels observed in first-pass organs, particularly the lungs (Huang *et al.*, *Nature Biotechnology*, 15:620 (1997); Templeton *et al.*, *Nature Biotechnology*, 15:647 (1997); Hofland *et al.*, *Pharmaceutical Research*, 14:742 (1997)).

[0010] Other liposomal delivery systems include, for example, the use of reverse micelles, anionic liposomes, and polymer liposomes. Reverse micelles are disclosed in U.S. Patent No. 6,429,200. Anionic liposomes are disclosed in U.S. Patent Publication No. 20030026831.

Polymer liposomes that incorporate dextrin or glycerol-phosphocholine polymers are disclosed in U.S. Patent Publication Nos. 20020081736 and 20030082103, respectively.

[0011] A gene delivery system containing an encapsulated nucleic acid for systemic delivery should be small (*i.e.*, less than about 100 nm diameter) and should remain intact in the circulation for an extended period of time in order to achieve delivery to affected tissues. This requires a highly stable, serum-resistant nucleic acid-containing particle that does not interact with cells and other components of the vascular compartment. The particle should also readily interact with target cells at a disease site in order to facilitate intracellular delivery of a desired nucleic acid.

[0012] Recent work has shown that nucleic acids can be encapsulated in small (*e.g.*, about 70 nm diameter) "stabilized plasmid-lipid particles" (SPLP) that consist of a single plasmid encapsulated within a bilayer lipid vesicle (Wheeler *et al.*, *Gene Therapy*, 6:271 (1999)). These SPLPs typically contain the "fusogenic" lipid dioleoylphosphatidylethanolamine (DOPE), low levels of cationic lipid, and are stabilized in aqueous media by the presence of a poly(ethylene glycol) (PEG) coating. SPLPs have systemic application as they exhibit extended circulation lifetimes following intravenous (i.v.) injection, accumulate preferentially at distal tumor sites due to the enhanced vascular permeability in such regions, and can mediate transgene expression at these tumor sites. The levels of transgene expression observed at the tumor site following i.v. injection of SPLPs containing the luciferase marker gene are superior to the levels that can be achieved employing plasmid DNA-cationic liposome complexes (lipoplexes) or naked DNA.

[0013] Thus, there remains a strong need in the art for novel and more efficient methods and compositions for introducing nucleic acids such as siRNA into cells. In addition, there is a need in the art for methods of downregulating the expression of genes of interest to treat or prevent diseases and disorders such as cancer and atherosclerosis. The present invention addresses these and other needs.

BRIEF SUMMARY OF THE INVENTION

[0014] The present invention provides novel, serum-stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (*e.g.*, for the treatment of a disease or disorder).

[0015] In preferred embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in

the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.*, by a nuclease or protease. In other preferred embodiments, the lipid particles are substantially non-toxic to mammals such as humans.

[0016] In one aspect, the present invention provides lipid particles comprising: (a) one or more active agents or therapeutic agents; (b) one or more cationic lipids comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0017] More particularly, the present invention provides serum-stable nucleic acid-lipid particles (SNALP) comprising a nucleic acid (*e.g.*, one or more interfering RNA molecules such as siRNA, aiRNA, and/or miRNA), methods of making the SNALP, and methods of delivering and/or administering the SNALP (*e.g.*, for the treatment of a disease or disorder).

[0018] In certain embodiments, the nucleic acid-lipid particle (*e.g.*, SNALP) comprises: (a) a nucleic acid (*e.g.*, an interfering RNA); (b) a cationic lipid comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0019] In one preferred embodiment, the nucleic acid-lipid particle (*e.g.*, SNALP) comprises: (a) an siRNA; (b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle; (c) cholesterol or a derivative thereof comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This preferred embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:62" formulation.

[0020] In another preferred embodiment, the nucleic acid-lipid particle (*e.g.*, SNALP) comprises: (a) an siRNA; (b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This preferred embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:57" formulation.

[0021] The present invention also provides pharmaceutical compositions comprising a lipid particle described herein (*e.g.*, SNALP) and a pharmaceutically acceptable carrier.

[0022] In another aspect, the present invention provides methods for introducing an active agent or therapeutic agent (*e.g.*, nucleic acid) into a cell, the method comprising contacting the cell with a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.*, SNALP).

[0023] In yet another aspect, the present invention provides methods for the *in vivo* delivery of an active agent or therapeutic agent (*e.g.*, nucleic acid), the method comprising administering to a mammalian subject a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.*, SNALP).

[0024] In a further aspect, the present invention provides methods for treating a disease or disorder in a mammalian subject in need thereof, the method comprising administering to the mammalian subject a therapeutically effective amount of a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.*, SNALP).

[0025] Other objects, features, and advantages of the present invention will be apparent to one of skill in the art from the following detailed description and figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] Figure 1A (Samples 1-8) and Figure 1B (Samples 9-16) illustrate data demonstrating the activity of 1:57 SNALP containing Eg5 siRNA in a human colon cancer cell line.

[0027] Figure 2 illustrates data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice.

[0028] Figure 3 illustrates additional data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice. Each bar represents the group mean of five animals. Error bars indicate the standard deviation.

[0029] Figure 4 illustrates data demonstrating the activity of 1:57 and 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

[0030] Figure 5 illustrates data demonstrating the activity of 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

[0031] Figure 6A (expressed as IU/L) and Figure 6B (expressed as x-Fold Upper Limit of Normal) illustrate data demonstrating that the tolerability of 1:57 SNALP containing ApoB siRNA prepared by citrate buffer versus PBS direct dilution did not differ significantly in terms of blood clinical chemistry parameters.

[0032] Figure 7A (expressed as liver ApoB:GAPD mRNA ratio), Figure 7B (expressed as relative plasma ApoB-100 concentration), and Figure 7C (expressed as plasma total cholesterol illustrate data demonstrating that the efficacy of 1:57 SNALP containing ApoB siRNA prepared by gear pump was similar to the same SNALP prepared by syringe press.

[0033] Figure 8 illustrates data demonstrating that there was very little effect on body weight 24 hours after administration of 1:57 SNALP containing ApoB siRNA.

[0034] Figure 9 illustrates data demonstrating that there were no obvious changes in platelet count after administration of 1:57 SNALP containing ApoB siRNA.

[0035] Figure 10A (expressed as IU/L) and Figure 10B (expressed as x-Fold Upper Limit of Normal) illustrate data demonstrating that clinically significant liver enzyme elevations (3xULN) occurred at particular drug dosages of 1:57 SNALP containing ApoB siRNA.

[0036] Figure 11A (expressed as liver ApoB:GAPD mRNA ratio) and Figure 11B (expressed as relative plasma ApoB-100 concentration) illustrate data demonstrating that the potency of the lower lipid:drug (L:D) 1:57 SNALP containing ApoB siRNA was as good as that of the higher L:D SNALP at the tested drug dosages.

[0037] Figure 12 illustrates data demonstrating that ApoB protein and total cholesterol levels were reduced to a similar extent by 1:57 SNALP containing ApoB siRNA at a 6:1 input L:D ratio (final ratio of 7:1) and 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1).

[0038] Figure 13 illustrates data demonstrating that a treatment regimen of 1:57 SNALP with siRNA targeting PLK-1 is well tolerated with no apparent signs of treatment related toxicity in mice bearing Hep3B liver tumors.

[0039] Figure 14 illustrates data demonstrating that treatment with 1:57 SNALP containing PLK-1 siRNA caused a significant increase in the survival of Hep3B tumor-bearing mice.

[0040] Figure 15 illustrates data demonstrating that treatment with 1:57 SNALP containing PLK-1 siRNA reduced PLK-1 mRNA levels by 50% in intrahepatic Hep3B tumors growing in mice 24 hours after SNALP administration.

[0041] Figure 16 illustrates data demonstrating that a specific cleavage product of PLK-1 mRNA was detectable by 5' RACE-PCR in mice treated with 1:57 SNALP containing PLK-1 siRNA. 10 μl PCR product/well were loaded onto a 1.5% agarose gel. Lane Nos.: (1) molecular weight (MW) marker; (2) PBS mouse 1; (3) PBS mouse 2; (4) PBS mouse 3; (5) Luc SNALP mouse 1; (6) Luc SNALP mouse 2; (7) PLK SNALP mouse 1; (8) PLK SNALP mouse 2; (9) PLK SNALP mouse 3; and (10) no template control.

[0042] Figure 17 illustrates data demonstrating that control (Luc) 1:57 SNALP-treated mice displayed normal mitoses in Hep3B tumors (top panels), whereas mice treated with 1:57 SNALP containing PLK-1 siRNA exhibited numerous aberrant mitoses and tumor cell apoptosis in Hep3B tumors (bottom panels).

[0043] Figure 18 illustrates data demonstrating that multiple doses of 1:57 PLK-1 SNALP containing PEG-cDSA induced the regression of established Hep3B subcutaneous (S.C.) tumors.

[0044] Figure 19 illustrates data demonstrating PLK-1 mRNA silencing using 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration.

[0045] Figure 20 illustrates data demonstrating that PLK-1 PEG-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

[0046] Figure 21 illustrates data demonstrating tumor-derived PLK-1 mRNA silencing in Hep3B intrahepatic tumors.

[0047] Figure 22 illustrates data demonstrating the blood clearance profile of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0048] The present invention is based, in part, upon the surprising discovery that lipid particles comprising from about 50 mol % to about 85 mol % of a cationic lipid, from about 13 mol % to about 49.5 mol % of a non-cationic lipid, and from about 0.5 mol % to about 2 mol % of a lipid conjugate provide advantages when used for the *in vitro* or *in vivo* delivery of an active agent, such as a therapeutic nucleic acid (*e.g.*, an interfering RNA). In particular, as illustrated by the Examples herein, the present invention provides stable nucleic acid-lipid particles (SNALP) that advantageously impart increased activity of the encapsulated nucleic acid (*e.g.*, an interfering RNA such as siRNA) and improved tolerability of the formulations *in vivo*, resulting in a significant increase in the therapeutic index as compared to nucleic acid-lipid particle compositions previously described. Additionally, the SNALP of the invention are stable in circulation, *e.g.*, resistant to degradation by nucleases in serum, and are substantially non-toxic to mammals such as humans. As a non-limiting example, Figure 3 of Example 4 shows that one SNALP embodiment of the invention ("1:57 SNALP") was more than 10 times as efficacious as compared to a nucleic acid-lipid particle previously described ("2:30 SNALP") in mediating target gene silencing at a 10-fold lower dose. Similarly, Figure 2 of Example 3 shows that the "1:57 SNALP" formulation was substantially

more effective at silencing the expression of a target gene as compared to nucleic acid-lipid particles previously described ("2:40 SNALP").

[0049] In certain embodiments, the present invention provides improved compositions for the delivery of interfering RNA such as siRNA molecules. In particular, the Examples herein illustrate that the improved lipid particle formulations of the invention are highly effective in downregulating the mRNA and/or protein levels of target genes. Furthermore, the Examples herein illustrate that the presence of certain molar ratios of lipid components results in improved or enhanced activity of these lipid particle formulations of the present invention. For instance, the "1:57 SNALP" and "1:62 SNALP" formulations described herein are exemplary formulations of the present invention that are particularly advantageous because they provide improved efficacy and tolerability *in vivo*, are serum-stable, are substantially non-toxic, are capable of accessing extravascular sites, and are capable of reaching target cell populations.

[0050] The lipid particles and compositions of the present invention may be used for a variety of purposes, including the delivery of associated or encapsulated therapeutic agents to cells, both *in vitro* and *in vivo*. Accordingly, the present invention provides methods for treating diseases or disorders in a subject in need thereof, by contacting the subject with a lipid particle described herein comprising one or more suitable therapeutic agents.

[0051] Various exemplary embodiments of the lipid particles of the invention, as well as compositions and formulations comprising the same, and their use to deliver therapeutic agents and modulate target gene and protein expression, are described in further detail below.

II. Definitions

[0052] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0053] The term "interfering RNA" or "RNAi" or "interfering RNA sequence" refers to single-stranded RNA (*e.g.*, mature miRNA) or double-stranded RNA (*i.e.*, duplex RNA such as siRNA, aiRNA, or pre-miRNA) that is capable of reducing or inhibiting the expression of a target gene or sequence (*e.g.*, by mediating the degradation or inhibiting the translation of mRNAs which are complementary to the interfering RNA sequence) when the interfering RNA is in the same cell as the target gene or sequence. Interfering RNA thus refers to the single-stranded RNA that is complementary to a target mRNA sequence or to the double-stranded RNA formed by two complementary strands or by a single, self-complementary strand. Interfering RNA may have substantial or complete identity to the target gene or

sequence, or may comprise a region of mismatch (*i.e.*, a mismatch motif). The sequence of the interfering RNA can correspond to the full-length target gene, or a subsequence thereof.

[0054] Interfering RNA includes "small-interfering RNA" or "siRNA," *e.g.*, interfering RNA of about 15-60, 15-50, or 15-40 (duplex) nucleotides in length, more typically about 15-30, 15-25, or 19-25 (duplex) nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 (duplex) nucleotides in length (*e.g.*, each complementary sequence of the double-stranded siRNA is 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, preferably about 20-24, 21-22, or 21-23 nucleotides in length, and the double-stranded siRNA is about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 base pairs in length, preferably about 18-22, 19-20, or 19-21 base pairs in length). siRNA duplexes may comprise 3' overhangs of about 1 to about 4 nucleotides or about 2 to about 3 nucleotides and 5' phosphate termini. Examples of siRNA include, without limitation, a double-stranded polynucleotide molecule assembled from two separate stranded molecules, wherein one strand is the sense strand and the other is the complementary antisense strand; a double-stranded polynucleotide molecule assembled from a single stranded molecule, where the sense and antisense regions are linked by a nucleic acid-based or non-nucleic acid-based linker; a double-stranded polynucleotide molecule with a hairpin secondary structure having self-complementary sense and antisense regions; and a circular single-stranded polynucleotide molecule with two or more loop structures and a stem having self-complementary sense and antisense regions, where the circular polynucleotide can be processed *in vivo* or *in vitro* to generate an active double-stranded siRNA molecule.

[0055] Preferably, siRNA are chemically synthesized. siRNA can also be generated by cleavage of longer dsRNA (*e.g.*, dsRNA greater than about 25 nucleotides in length) with the *E. coli* RNase III or Dicer. These enzymes process the dsRNA into biologically active siRNA (*see, e.g.*, Yang *et al.*, *Proc. Natl. Acad. Sci. USA,* 99:9942-9947 (2002); Calegari *et al.*, *Proc. Natl. Acad. Sci. USA,* 99:14236 (2002); Byrom *et al.*, *Ambion TechNotes,* 10(1):4-6 (2003); Kawasaki *et al.*, *Nucleic Acids Res.,* 31:981-987 (2003); Knight *et al.*, *Science,* 293:2269-2271 (2001); and Robertson *et al.*, *J. Biol. Chem.,* 243:82 (1968)). Preferably, dsRNA are at least 50 nucleotides to about 100, 200, 300, 400, or 500 nucleotides in length. A dsRNA may be as long as 1000, 1500, 2000, 5000 nucleotides in length, or longer. The dsRNA can encode for an entire gene transcript or a partial gene transcript. In certain instances, siRNA may be encoded by a plasmid (*e.g.*, transcribed as sequences that automatically fold into duplexes with hairpin loops).

[0056] As used herein, the term "mismatch motif" or "mismatch region" refers to a portion of an interfering RNA (*e.g.*, siRNA, aiRNA, miRNA) sequence that does not have 100 % complementarity to its target sequence. An interfering RNA may have at least one, two, three, four, five, six, or more mismatch regions. The mismatch regions may be contiguous or may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides. The mismatch motifs or regions may comprise a single nucleotide or may comprise two, three, four, five, or more nucleotides.

[0057] An "effective amount" or "therapeutically effective amount" of an active agent or therapeutic agent such as an interfering RNA is an amount sufficient to produce the desired effect, *e.g.*, an inhibition of expression of a target sequence in comparison to the normal expression level detected in the absence of an interfering RNA. Inhibition of expression of a target gene or target sequence is achieved when the value obtained with an interfering RNA relative to the control is about 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. Suitable assays for measuring expression of a target gene or target sequence include, *e.g.*, examination of protein or RNA levels using techniques known to those of skill in the art such as dot blots, northern blots, *in situ* hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

[0058] By "decrease," "decreasing," "reduce," or "reducing" of an immune response by an interfering RNA is intended to mean a detectable decrease of an immune response to a given interfering RNA (*e.g.*, a modified interfering RNA). The amount of decrease of an immune response by a modified interfering RNA may be determined relative to the level of an immune response in the presence of an unmodified interfering RNA. A detectable decrease can be about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, or more lower than the immune response detected in the presence of the unmodified interfering RNA. A decrease in the immune response to interfering RNA is typically measured by a decrease in cytokine production (*e.g.*, IFNγ, IFNα, TNFα, IL-6, or IL-12) by a responder cell *in vitro* or a decrease in cytokine production in the sera of a mammalian subject after administration of the interfering RNA.

[0059] As used herein, the term "responder cell" refers to a cell, preferably a mammalian cell, that produces a detectable immune response when contacted with an immunostimulatory interfering RNA such as an unmodified siRNA. Exemplary responder cells include, *e.g.*, dendritic cells, macrophages, peripheral blood mononuclear cells (PBMCs), splenocytes, and the like. Detectable immune responses include, *e.g.*, production of cytokines or growth

factors such as TNF-α, IFN-α, IFN-β, IFN-γ, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TGF, and combinations thereof.

[0060] "Substantial identity" refers to a sequence that hybridizes to a reference sequence under stringent conditions, or to a sequence that has a specified percent identity over a specified region of a reference sequence.

[0061] The phrase "stringent hybridization conditions" refers to conditions under which a nucleic acid will hybridize to its target sequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization.

[0062] Exemplary stringent hybridization conditions can be as follows: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C. For PCR, a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90°C-95°C for 30 sec.-2 min., an annealing phase lasting 30 sec.-2 min., and an extension phase of about 72°C for 1-2 min. Protocols and guidelines for low and high stringency amplification reactions are provided, *e.g.*, in Innis *et al.*, *PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc. N.Y. (1990).

[0063] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional guidelines for determining hybridization parameters are provided in numerous references, *e.g.*, Current Protocols in Molecular Biology, Ausubel *et al.*, eds.

[0064] The terms "substantially identical" or "substantial identity," in the context of two or more nucleic acids, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides that are the same (*i.e.*, at least about 60%, preferably at least about 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition, when the context indicates, also refers analogously to the complement of a sequence. Preferably, the substantial identity exists over a region that is at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 nucleotides in length.

[0065] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0066] A "comparison window," as used herein, includes reference to a segment of any one of a number of contiguous positions selected from the group consisting of from about 5 to about 60, usually about 10 to about 45, more usually about 15 to about 30, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences

for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.*, 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.*, 48:443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA*, 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g., Current Protocols in Molecular Biology*, Ausubel *et al.*, eds. (1995 supplement)).

[0067] A preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al., Nuc. Acids Res.*, 25:3389-3402 (1977) and Altschul *et al., J. Mol. Biol.*, 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

[0068] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin and Altschul, *Proc. Natl. Acad. Sci. USA*, 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0069] The term "nucleic acid" as used herein refers to a polymer containing at least two deoxyribonucleotides or ribonucleotides in either single- or double-stranded form and includes DNA and RNA. DNA may be in the form of, *e.g.*, antisense molecules, plasmid DNA, pre-condensed DNA, a PCR product, vectors (P1, PAC, BAC, YAC, artificial chromosomes), expression cassettes, chimeric sequences, chromosomal DNA, or derivatives and combinations of these groups. RNA may be in the form of siRNA, asymmetrical interfering RNA (aiRNA), microRNA (miRNA), mRNA, tRNA, rRNA, tRNA, viral RNA (vRNA), and combinations thereof. Nucleic acids include nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, and which have similar binding properties as the

reference nucleic acid. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2'-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs). Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.*, 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.*, 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes*, 8:91-98 (1994)). "Nucleotides" contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and natural analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides.

[0070] The term "gene" refers to a nucleic acid (*e.g.*, DNA or RNA) sequence that comprises partial length or entire length coding sequences necessary for the production of a polypeptide or precursor polypeptide.

[0071] "Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

[0072] The term "lipid" refers to a group of organic compounds that include, but are not limited to, esters of fatty acids and are characterized by being insoluble in water, but soluble in many organic solvents. They are usually divided into at least three classes: (1) "simple lipids," which include fats and oils as well as waxes; (2) "compound lipids," which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids.

[0073] A "lipid particle" is used herein to refer to a lipid formulation that can be used to deliver an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, an interfering RNA), to a target site of interest. In the lipid particle of the invention, which is typically formed from a cationic lipid, a non-cationic lipid, and a conjugated lipid that prevents aggregation of

the particle, the active agent or therapeutic agent may be encapsulated in the lipid, thereby protecting the agent from enzymatic degradation.

[0074] As used herein, the term "SNALP" refers to a stable nucleic acid-lipid particle. A SNALP represents a particle made from lipids (*e.g.*, a cationic lipid, a non-cationic lipid, and a conjugated lipid that prevents aggregation of the particle), wherein the nucleic acid (*e.g.*, siRNA, aiRNA, miRNA, ssDNA, dsDNA, ssRNA, short hairpin RNA (shRNA), dsRNA, or a plasmid, including plasmids from which an interfering RNA is transcribed) is fully encapsulated within the lipid. As used herein, the term "SNALP" includes an SPLP, which is the term used to refer to a nucleic acid-lipid particle comprising a nucleic acid (*e.g.*, a plasmid) encapsulated within the lipid. SNALP and SPLP typically contain a cationic lipid, a non-cationic lipid, and a lipid conjugate (*e.g.*, a PEG-lipid conjugate). SNALP and SPLP are extremely useful for systemic applications, as they can exhibit extended circulation lifetimes following intravenous (i.v.) injection, they can accumulate at distal sites (*e.g.*, sites physically separated from the administration site), and they can mediate expression of the transfected gene or silencing of target gene expression at these distal sites. SPLP include "pSPLP," which comprise an encapsulated condensing agent-nucleic acid complex as set forth in PCT Publication No. WO 00/03683, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0075] The lipid particles of the invention (*e.g.*, SNALP) typically have a mean diameter of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm, and are substantially non-toxic. In addition, nucleic acids, when present in the lipid particles of the invention, are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.*, U.S. Patent Publication Nos. 20040142025 and 20070042031, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0076] As used herein, "lipid encapsulated" can refer to a lipid particle that provides an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, an interfering RNA), with full encapsulation, partial encapsulation, or both. In a preferred embodiment, the nucleic acid is fully encapsulated in the lipid particle (*e.g.*, to form an SPLP, pSPLP, SNALP, or other nucleic acid-lipid particle).

[0077] The term "lipid conjugate" refers to a conjugated lipid that inhibits aggregation of lipid particles. Such lipid conjugates include, but are not limited to, polyamide oligomers (*e.g.*, ATTA-lipid conjugates), PEG-lipid conjugates, such as PEG coupled to

dialkyloxypropyls, PEG coupled to diacylglycerols, PEG coupled to cholesterol, PEG coupled to phosphatidylethanolamines, PEG conjugated to ceramides (*see, e.g.,* U.S. Patent No. 5,885,613, the disclosure of which is herein incorporated by reference in its entirety for all purposes), cationic PEG lipids, and mixtures thereof. PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.,* non-ester containing linker moieties and ester-containing linker moieties. In preferred embodiments, non-ester containing linker moieties are used.

[0078] The term "amphipathic lipid" refers, in part, to any suitable material wherein the hydrophobic portion of the lipid material orients into a hydrophobic phase, while the hydrophilic portion orients toward the aqueous phase. Hydrophilic characteristics derive from the presence of polar or charged groups such as carbohydrates, phosphate, carboxylic, sulfato, amino, sulfhydryl, nitro, hydroxyl, and other like groups. Hydrophobicity can be conferred by the inclusion of apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Examples of amphipathic compounds include, but are not limited to, phospholipids, aminolipids, and sphingolipids.

[0079] Representative examples of phospholipids include, but are not limited to, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine. Other compounds lacking in phosphorus, such as sphingolipid, glycosphingolipid families, diacylglycerols, and β-acyloxyacids, are also within the group designated as amphipathic lipids. Additionally, the amphipathic lipids described above can be mixed with other lipids including triglycerides and sterols.

[0080] The term "neutral lipid" refers to any of a number of lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cholesterol, cerebrosides, and diacylglycerols.

[0081] The term "non-cationic lipid" refers to any amphipathic lipid as well as any other neutral lipid or anionic lipid.

[0082] The term "anionic lipid" refers to any lipid that is negatively charged at physiological pH. These lipids include, but are not limited to, phosphatidylglycerols, cardiolipins, diacylphosphatidylserines, diacylphosphatidic acids, N-dodecanoyl phosphatidylethanolamines, N-succinyl phosphatidylethanolamines, N-glutarylphosphatidylethanolamines, lysylphosphatidylglycerols, palmitoyloleyolphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

[0083] The term "cationic lipid" refers to any of a number of lipid species that carry a net positive charge at a selected pH, such as physiological pH (*e.g.*, pH of about 7.0). It has been surprisingly found that cationic lipids comprising alkyl chains with multiple sites of unsaturation, *e.g.*, at least two or three sites of unsaturation, are particularly useful for forming lipid particles with increased membrane fluidity. A number of cationic lipids and related analogs, which are also useful in the present invention, have been described in U.S. Patent Publication Nos. 20060083780 and 20060240554; U.S. Patent Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390, the disclosures of which are herein incorporated by reference in their entirety for all purposes. Non-limiting examples of cationic lipids are described in detail herein. In some cases, the cationic lipids comprise a protonatable tertiary amine (*e.g.*, pH titratable) head group, C18 alkyl chains, ether linkages between the head group and alkyl chains, and 0 to 3 double bonds. Such lipids include, *e.g.*, DSDMA, DLinDMA, DLenDMA, and DODMA.

[0084] The term "hydrophobic lipid" refers to compounds having apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups optionally substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Suitable examples include, but are not limited to, diacylglycerol, dialkylglycerol, N-N-dialkylamino, 1,2-diacyloxy-3-aminopropane, and 1,2-dialkyl-3-aminopropane.

[0085] The term "fusogenic" refers to the ability of a lipid particle, such as a SNALP, to fuse with the membranes of a cell. The membranes can be either the plasma membrane or membranes surrounding organelles, *e.g.*, endosome, nucleus, *etc.*

[0086] As used herein, the term "aqueous solution" refers to a composition comprising in whole, or in part, water.

[0087] As used herein, the term "organic lipid solution" refers to a composition comprising in whole, or in part, an organic solvent having a lipid.

[0088] "Distal site," as used herein, refers to a physically separated site, which is not limited to an adjacent capillary bed, but includes sites broadly distributed throughout an organism.

[0089] "Serum-stable" in relation to nucleic acid-lipid particles such as SNALP means that the particle is not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA or RNA. Suitable assays include, for example, a standard serum assay, a DNAse assay, or an RNAse assay.

[0090] "Systemic delivery," as used herein, refers to delivery of lipid particles that leads to a broad biodistribution of an active agent or therapeutic agent such as an interfering RNA within an organism. Some techniques of administration can lead to the systemic delivery of certain agents, but not others. Systemic delivery means that a useful, preferably therapeutic, amount of an agent is exposed to most parts of the body. To obtain broad biodistribution generally requires a blood lifetime such that the agent is not rapidly degraded or cleared (such as by first pass organs (liver, lung, *etc.*) or by rapid, nonspecific cell binding) before reaching a disease site distal to the site of administration. Systemic delivery of lipid particles can be by any means known in the art including, for example, intravenous, subcutaneous, and intraperitoneal. In a preferred embodiment, systemic delivery of lipid particles is by intravenous delivery.

[0091] "Local delivery," as used herein, refers to delivery of an active agent or therapeutic agent such as an interfering RNA directly to a target site within an organism. For example, an agent can be locally delivered by direct injection into a disease site such as a tumor or other target site such as a site of inflammation or a target organ such as the liver, heart, pancreas, kidney, and the like.

[0092] The term "mammal" refers to any mammalian species such as a human, mouse, rat, dog, cat, hamster, guinea pig, rabbit, livestock, and the like.

[0093] The term "cancer" refers to any member of a class of diseases characterized by the uncontrolled growth of aberrant cells. The term includes all known cancers and neoplastic conditions, whether characterized as malignant, benign, soft tissue, or solid, and cancers of all stages and grades including pre- and post-metastatic cancers. Examples of different types of cancer include, but are not limited to, lung cancer, colon cancer, rectal cancer, anal cancer, bile duct cancer, small intestine cancer, stomach (gastric) cancer, esophageal cancer; gallbladder cancer, liver cancer, pancreatic cancer, appendix cancer, breast cancer, ovarian cancer; cervical cancer, prostate cancer, renal cancer (*e.g.*, renal cell carcinoma), cancer of the central nervous system, glioblastoma, skin cancer, lymphomas, choriocarcinomas, head

and neck cancers, osteogenic sarcomas, and blood cancers. Non-limiting examples of specific types of liver cancer include hepatocellular carcinoma (HCC), secondary liver cancer (*e.g.*, caused by metastasis of some other non-liver cancer cell type), and hepatoblastoma. As used herein, a "tumor" comprises one or more cancerous cells.

III. Description of the Embodiments

[0094] The present invention provides novel, serum-stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (*e.g.*, for the treatment of a disease or disorder).

[0095] In one aspect, the present invention provides lipid particles comprising: (a) one or more active agents or therapeutic agents; (b) one or more cationic lipids comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0096] In certain embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.*, by a nuclease or protease. In certain other embodiments, the lipid particles are substantially non-toxic to mammals such as humans.

[0097] In some embodiments, the active agent or therapeutic agent comprises a nucleic acid. In certain instances, the nucleic acid comprises an interfering RNA molecule such as, *e.g.*, an siRNA, aiRNA, miRNA, or mixtures thereof. In certain other instances, the nucleic acid comprises single-stranded or double-stranded DNA, RNA, or a DNA/RNA hybrid such as, *e.g.*, an antisense oligonucleotide, a ribozyme, a plasmid, an immunostimulatory oligonucleotide, or mixtures thereof.

[0098] In other embodiments, the active agent or therapeutic agent comprises a peptide or polypeptide. In certain instances, the peptide or polypeptide comprises an antibody such as, *e.g.*, a polyclonal antibody, a monoclonal antibody, an antibody fragment; a humanized antibody, a recombinant antibody, a recombinant human antibody, a Primatized™ antibody, or mixtures thereof. In certain other instances, the peptide or polypeptide comprises a cytokine, a growth factor, an apoptotic factor, a differentiation-inducing factor, a cell-surface

receptor, a ligand, a hormone, a small molecule (*e.g.*, small organic molecule or compound), or mixtures thereof.

[0099] In preferred embodiments, the active agent or therapeutic agent comprises an siRNA. In one embodiment, the siRNA molecule comprises a double-stranded region of about 15 to about 60 nucleotides in length (*e.g.*, about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, or 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length). The siRNA molecules of the invention are capable of silencing the expression of a target sequence *in vitro* and/or *in vivo*.

[0100] In some embodiments, the siRNA molecule comprises at least one modified nucleotide. In certain preferred embodiments, the siRNA molecule comprises one, two, three, four, five, six, seven, eight, nine, ten, or more modified nucleotides in the double-stranded region. In certain instances, the siRNA comprises from about 1% to about 100% (*e.g.*, about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) modified nucleotides in the double-stranded region. In preferred embodiments, less than about 25% (*e.g.*, less than about 25%, 20%, 15%, 10%, or 5%) or from about 1% to about 25% (*e.g.*, from about 1%-25%, 5%-25%, 10%-25%, 15%-25%, 20%-25%, or 10%-20%) of the nucleotides in the double-stranded region comprise modified nucleotides.

[0101] In other embodiments, the siRNA molecule comprises modified nucleotides including, but not limited to, 2'-O-methyl (2'OMe) nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, locked nucleic acid (LNA) nucleotides, and mixtures thereof. In preferred embodiments, the siRNA comprises 2'OMe nucleotides (*e.g.*, 2'OMe purine and/or pyrimidine nucleotides) such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, 2'OMe-cytosine nucleotides, and mixtures thereof. In certain instances, the siRNA does not comprise 2'OMe-cytosine nucleotides. In other embodiments, the siRNA comprises a hairpin loop structure.

[0102] The siRNA may comprise modified nucleotides in one strand (*i.e.*, sense or antisense) or both strands of the double-stranded region of the siRNA molecule. Preferably, uridine and/or guanosine nucleotides are modified at selective positions in the double-stranded region of the siRNA duplex. With regard to uridine nucleotide modifications, at least one, two, three, four, five, six, or more of the uridine nucleotides in the sense and/or antisense strand can be a modified uridine nucleotide such as a 2'OMe-uridine nucleotide. In some embodiments, every uridine nucleotide in the sense and/or antisense strand is a 2'OMe-

uridine nucleotide. With regard to guanosine nucleotide modifications, at least one, two, three, four, five, six, or more of the guanosine nucleotides in the sense and/or antisense strand can be a modified guanosine nucleotide such as a 2'OMe-guanosine nucleotide. In some embodiments, every guanosine nucleotide in the sense and/or antisense strand is a 2'OMe-guanosine nucleotide.

[0103] In certain embodiments, at least one, two, three, four, five, six, seven, or more 5'-GU-3' motifs in an siRNA sequence may be modified, *e.g.*, by introducing mismatches to eliminate the 5'-GU-3' motifs and/or by introducing modified nucleotides such as 2'OMe nucleotides. The 5'-GU-3' motif can be in the sense strand, the antisense strand, or both strands of the siRNA sequence. The 5'-GU-3' motifs may be adjacent to each other or, alternatively, they may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides.

[0104] In some preferred embodiments, a modified siRNA molecule is less immunostimulatory than a corresponding unmodified siRNA sequence. In such embodiments, the modified siRNA molecule with reduced immunostimulatory properties advantageously retains RNAi activity against the target sequence. In another embodiment, the immunostimulatory properties of the modified siRNA molecule and its ability to silence target gene expression can be balanced or optimized by the introduction of minimal and selective 2'OMe modifications within the siRNA sequence such as, *e.g.*, within the double-stranded region of the siRNA duplex. In certain instances, the modified siRNA is at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% less immunostimulatory than the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that the immunostimulatory properties of the modified siRNA molecule and the corresponding unmodified siRNA molecule can be determined by, for example, measuring INF-α and/or IL-6 levels from about two to about twelve hours after systemic administration in a mammal or transfection of a mammalian responder cell using an appropriate lipid-based delivery system (such as the SNALP delivery system disclosed herein).

[0105] In certain embodiments, a modified siRNA molecule has an IC_{50} (*i.e.*, half-maximal inhibitory concentration) less than or equal to ten-fold that of the corresponding unmodified siRNA (*i.e.*, the modified siRNA has an IC_{50} that is less than or equal to ten-times the IC_{50} of the corresponding unmodified siRNA). In other embodiments, the modified siRNA has an IC_{50} less than or equal to three-fold that of the corresponding unmodified siRNA sequence. In yet other embodiments, the modified siRNA has an IC_{50} less than or equal to two-fold that

of the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that a dose-response curve can be generated and the IC_{50} values for the modified siRNA and the corresponding unmodified siRNA can be readily determined using methods known to those of skill in the art.

[0106] In yet another embodiment, a modified siRNA molecule is capable of silencing at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the expression of the target sequence relative to the corresponding unmodified siRNA sequence.

[0107] In some embodiments, the siRNA molecule does not comprise phosphate backbone modifications, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In other embodiments, the siRNA comprises one, two, three, four, or more phosphate backbone modifications, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise phosphate backbone modifications.

[0108] In further embodiments, the siRNA does not comprise 2'-deoxy nucleotides, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In yet further embodiments, the siRNA comprises one, two, three, four, or more 2'-deoxy nucleotides, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise 2'-deoxy nucleotides.

[0109] In certain instances, the nucleotide at the 3'-end of the double-stranded region in the sense and/or antisense strand is not a modified nucleotide. In certain other instances, the nucleotides near the 3'-end (*e.g.*, within one, two, three, or four nucleotides of the 3'-end) of the double-stranded region in the sense and/or antisense strand are not modified nucleotides.

[0110] The siRNA molecules described herein may have 3' overhangs of one, two, three, four, or more nucleotides on one or both sides of the double-stranded region, or may lack overhangs (*i.e.*, have blunt ends) on one or both sides of the double-stranded region. Preferably, the siRNA has 3' overhangs of two nucleotides on each side of the double-stranded region. In certain instances, the 3' overhang on the antisense strand has complementarity to the target sequence and the 3' overhang on the sense strand has complementarity to a complementary strand of the target sequence. Alternatively, the 3' overhangs do not have complementarity to the target sequence or the complementary strand thereof. In some embodiments, the 3' overhangs comprise one, two, three, four, or more nucleotides such as 2'-deoxy (2'H) nucleotides. In certain preferred embodiments, the 3' overhangs comprise deoxythymidine (dT) and/or uridine nucleotides. In other embodiments, one or more of the nucleotides in the 3' overhangs on one or both sides of the double-

stranded region comprise modified nucleotides. Non-limiting examples of modified nucleotides are described above and include 2'OMe nucleotides, 2'-deoxy-2'F nucleotides, 2'-deoxy nucleotides, 2'-O-2-MOE nucleotides, LNA nucleotides, and mixtures thereof. In preferred embodiments, one, two, three, four, or more nucleotides in the 3' overhangs present on the sense and/or antisense strand of the siRNA comprise 2'OMe nucleotides (*e.g.*, 2'OMe purine and/or pyrimidine nucleotides) such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, 2'OMe-cytosine nucleotides, and mixtures thereof.

[0111] The siRNA may comprise at least one or a cocktail (*e.g.*, at least two, three, four, five, six, seven, eight, nine, ten, or more) of unmodified and/or modified siRNA sequences that silence target gene expression. The cocktail of siRNA may comprise sequences which are directed to the same region or domain (*e.g.*, a "hot spot") and/or to different regions or domains of one or more target genes. In certain instances, one or more (*e.g.*, at least two, three, four, five, six, seven, eight, nine, ten, or more) modified siRNA that silence target gene expression are present in a cocktail. In certain other instances, one or more (*e.g.*, at least two, three, four, five, six, seven, eight, nine, ten, or more) unmodified siRNA sequences that silence target gene expression are present in a cocktail.

[0112] In some embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% complementary to the target sequence or a portion thereof. In other embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that is 100% complementary to the target sequence or a portion thereof. In further embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that specifically hybridizes to the target sequence or a portion thereof.

[0113] In further embodiments, the sense strand of the siRNA molecule comprises or consists of a sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the target sequence or a portion thereof. In additional embodiments, the sense strand of the siRNA molecule comprises or consists of a sequence that is 100% identical to the target sequence or a portion thereof.

[0114] In the lipid particles of the invention (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the cationic lipid may comprise, *e.g.*, one or more of the following: 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-

dioxolane (DLin-K-C3-DMA), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3 -(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',1-2'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), or mixtures thereof. In certain preferred embodiments, the cationic lipid is DLinDMA, DLin-K-C2-DMA ("XTC2"), or mixtures thereof.

[0115] The synthesis of cationic lipids such as DLin-K-C2-DMA ("XTC2"), DLin-K-C3-DMA, DLin-K-C4-DMA, DLin-K6-DMA, and DLin-K-MPZ, as well as additional cationic lipids, is described in U.S. Provisional Application No. 61/104,212, filed October 9, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes. The synthesis of cationic lipids such as DLin-K-DMA, DLin-C-DAP, DLin-DAC, DLin-MA, DLinDAP, DLin-S-DMA, DLin-2-DMAP, DLin-TMA.Cl, DLin-TAP.Cl, DLin-MPZ,

DLinAP, DOAP, and DLin-EG-DMA, as well as additional cationic lipids, is described in PCT Application No. PCT/US08/88676, filed December 31, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes. The synthesis of cationic lipids such as CLinDMA, as well as additional cationic lipids, is described in U.S. Patent Publication No. 20060240554, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0116] In some embodiments, the cationic lipid may comprise from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, or from about 50 mol % to about 60 mol % of the total lipid present in the particle.

[0117] In other embodiments, the cationic lipid may comprise from about 55 mol % to about 90 mol %, from about 55 mol % to about 85 mol %, from about 55 mol % to about 80 mol %, from about 55 mol % to about 75 mol %, from about 55 mol % to about 70 mol %, or from about 55 mol % to about 65 mol % of the total lipid present in the particle.

[0118] In yet other embodiments, the cationic lipid may comprise from about 60 mol % to about 90 mol %, from about 60 mol % to about 85 mol %, from about 60 mol % to about 80 mol %, from about 60 mol % to about 75 mol %, or from about 60 mol % to about 70 mol % of the total lipid present in the particle.

[0119] In still yet other embodiments, the cationic lipid may comprise from about 65 mol % to about 90 mol %, from about 65 mol % to about 85 mol %, from about 65 mol % to about 80 mol %, or from about 65 mol % to about 75 mol % of the total lipid present in the particle.

[0120] In further embodiments, the cationic lipid may comprise from about 70 mol % to about 90 mol %, from about 70 mol % to about 85 mol %, from about 70 mol % to about 80 mol %, from about 75 mol % to about 90 mol %, from about 75 mol % to about 85 mol %, or from about 80 mol % to about 90 mol % of the total lipid present in the particle.

[0121] In additional embodiments, the cationic lipid may comprise (at least) about 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, or 90 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0122] In the lipid particles of the invention (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the non-cationic lipid may comprise, *e.g.*, one or more anionic lipids and/or neutral lipids. In preferred embodiments, the non-cationic lipid comprises one of the

following neutral lipid components: (1) cholesterol or a derivative thereof; (2) a phospholipid; or (3) a mixture of a phospholipid and cholesterol or a derivative thereof.

[0123] Examples of cholesterol derivatives include, but are not limited to, cholestanol, cholestanone, cholestenone, coprostanol, cholesteryl-2'-hydroxyethyl ether, cholesteryl-4'-hydroxybutyl ether, and mixtures thereof. The synthesis of cholesteryl-2'-hydroxyethyl ether is described herein.

[0124] The phospholipid may be a neutral lipid including, but not limited to, dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol (POPG), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), egg phosphatidylcholine (EPC), and mixtures thereof. In certain preferred embodiments, the phospholipid is DPPC, DSPC, or mixtures thereof.

[0125] In some embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 60 mol %, from about 15 mol % to about 60 mol %, from about 20 mol % to about 60 mol %, from about 25 mol % to about 60 mol %, from about 30 mol % to about 60 mol %, from about 10 mol % to about 55 mol %, from about 15 mol % to about 55 mol %, from about 20 mol % to about 55 mol %, from about 25 mol % to about 55 mol %, from about 30 mol % to about 55 mol %, from about 13 mol % to about 50 mol %, from about 15 mol % to about 50 mol % or from about 20 mol % to about 50 mol % of the total lipid present in the particle. When the non-cationic lipid is a mixture of a phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40, 50, or 60 mol % of the total lipid present in the particle.

[0126] In other embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 49.5 mol %, from about 13 mol % to about 49.5 mol %, from about 15 mol % to about 49.5 mol %, from about 20 mol % to about 49.5 mol %, from about 25 mol % to about 49.5 mol %, from about 30 mol % to about 49.5 mol %, from about 35 mol % to about 49.5 mol %, or from about 40 mol % to about 49.5 mol % of the total lipid present in the particle.

[0127] In yet other embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 45 mol %, from about 13

mol % to about 45 mol %, from about 15 mol % to about 45 mol %, from about 20 mol % to about 45 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, or from about 35 mol % to about 45 mol % of the total lipid present in the particle.

[0128] In still yet other embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 40 mol %, from about 13 mol % to about 40 mol %, from about 15 mol % to about 40 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 40 mol %, or from about 30 mol % to about 40 mol % of the total lipid present in the particle.

[0129] In further embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 35 mol %, from about 13 mol % to about 35 mol %, from about 15 mol % to about 35 mol %, from about 20 mol % to about 35 mol %, or from about 25 mol % to about 35 mol % of the total lipid present in the particle.

[0130] In yet further embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 30 mol %, from about 13 mol % to about 30 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 13 mol % to about 25 mol %, or from about 15 mol % to about 25 mol % of the total lipid present in the particle.

[0131] In additional embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise (at least) about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0132] In certain preferred embodiments, the non-cationic lipid comprises cholesterol or a derivative thereof of from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle. As a non-limiting example, a phospholipid-free lipid particle of the invention may comprise cholesterol or a derivative thereof at about 37 mol % of the total lipid present in the particle. In other preferred embodiments, a phospholipid-free lipid particle of the invention may comprise cholesterol or a derivative thereof of from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 45 mol %, from about 40 mol % to about 45 mol %, from about 32 mol % to about 45 mol %, from about 32 mol % to about 42 mol %, from about 32 mol % to about 40 mol %, from about 34 mol % to about 45 mol %, from about 34 mol % to about 42 mol %, from about 34 mol % to about 40 mol %, or about 30, 31, 32, 33, 34, 35, 36,

37, 38, 39, 40, 41, 42, 43, 44, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0133] In certain other preferred embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 4 mol % to about 10 mol % of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 30 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of a phospholipid and cholesterol may comprise DPPC at about 7 mol % and cholesterol at about 34 mol % of the total lipid present in the particle. In other embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 3 mol % to about 15 mol %, from about 4 mol % to about 15 mol %, from about 4 mol % to about 12 mol %, from about 4 mol % to about 10 mol %, from about 4 mol % to about 8 mol %, from about 5 mol % to about 12 mol %, from about 5 mol % to about 9 mol %, from about 6 mol % to about 12 mol %, from about 6 mol % to about 10 mol %, or about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 mol % (or any fraction thereof or range therein) of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 40 mol %, from about 25 mol % to about 35 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 45 mol %, from about 40 mol % to about 45 mol %, from about 28 mol % to about 40 mol %, from about 28 mol % to about 38 mol %, from about 30 mol % to about 38 mol %, from about 32 mol % to about 36 mol %, or about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0134] In further preferred embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 10 mol % to about 30 mol % of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 10 mol % to about 30 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of a phospholipid and cholesterol may comprise DPPC at about 20 mol % and cholesterol at about 20 mol % of the total lipid present in the particle. In other embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 10 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 10 mol % to about 20 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, from about 12 mol % to about 28 mol %, from about 14 mol % to about 26 mol %, or about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20,

21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 mol % (or any fraction thereof or range therein) of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 10 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 10 mol % to about 20 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, from about 12 mol % to about 28 mol %, from about 14 mol % to about 26 mol %, or about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0135] In the lipid particles of the invention (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the conjugated lipid that inhibits aggregation of particles may comprise, *e.g.*, one or more of the following: a polyethyleneglycol (PEG)-lipid conjugate, a polyamide (ATTA)-lipid conjugate, a cationic-polymer-lipid conjugates (CPLs), or mixtures thereof. In one preferred embodiment, the nucleic acid-lipid particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate. In certain embodiments, the PEG-lipid conjugate or ATTA-lipid conjugate is used together with a CPL. The conjugated lipid that inhibits aggregation of particles may comprise a PEG-lipid including, *e.g.*, a PEG-diacylglycerol (DAG), a PEG dialkyloxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or mixtures thereof. The PEG-DAA conjugate may be PEG-dilauryloxypropyl (C12), a PEG-dimyristyloxypropyl (C14), a PEG-dipalmityloxypropyl (C16), a PEG-distearyloxypropyl (C18), or mixtures thereof.

[0136] Additional PEG-lipid conjugates suitable for use in the invention include, but are not limited to, mPEG2000-1,2-di-O-alkyl-*sn*3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Application No. PCT/US08/88676, filed December 31, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Yet additional PEG-lipid conjugates suitable for use in the invention include, without limitation, 1-[8'-(1,2-dimyristoyl-3-propanoxy)-carboxamido-3',6'-dioxaoctanyl]carbamoyl-ω-methyl-poly(ethylene glycol) (2KPEG-DMG). The synthesis of 2KPEG-DMG is described in U.S. Patent No. 7,404,969, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0137] The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about

750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

[0138] In some embodiments, the conjugated lipid that inhibits aggregation of particles is a CPL that has the formula: A-W-Y, wherein A is a lipid moiety, W is a hydrophilic polymer, and Y is a polycationic moiety. W may be a polymer selected from the group consisting of polyethyleneglycol (PEG), polyamide, polylactic acid, polyglycolic acid, polylactic acid/polyglycolic acid copolymers, or combinations thereof, the polymer having a molecular weight of from about 250 to about 7000 daltons. In some embodiments, Y has at least 4 positive charges at a selected pH. In some embodiments, Y may be lysine, arginine, asparagine, glutamine, derivatives thereof, or combinations thereof.

[0139] In certain instances, the conjugated lipid that inhibits aggregation of particles (*e.g.*, PEG-lipid conjugate) may comprise from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 0.6 mol % to about 1.9 mol %, from about 0.7 mol % to about 1.8 mol %, from about 0.8 mol % to about 1.7 mol %, from about 1 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.7 mol %, from about 1.3 mol % to about 1.6 mol %, from about 1.4 mol % to about 1.5 mol %, or about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0140] In the lipid particles of the invention, the active agent or therapeutic agent may be fully encapsulated within the lipid portion of the particle, thereby protecting the active agent or therapeutic agent from enzymatic degradation. In preferred embodiments, a SNALP comprising a nucleic acid such as an interfering RNA (*e.g.*, siRNA) is fully encapsulated within the lipid portion of the particle, thereby protecting the nucleic acid from nuclease degradation. In certain instances, the nucleic acid in the SNALP is not substantially degraded after exposure of the particle to a nuclease at 37°C for at least about 20, 30, 45, or 60 minutes. In certain other instances, the nucleic acid in the SNALP is not substantially degraded after incubation of the particle in serum at 37°C for at least about 30, 45, or 60 minutes or at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, or 36 hours. In other embodiments, the active agent or therapeutic agent (*e.g.*, nucleic acid such as siRNA) is complexed with the lipid portion of the particle. One of the benefits of the formulations of the present invention is that the lipid particle compositions are substantially non-toxic to mammals such as humans.

[0141] The term "fully encapsulated" indicates that the active agent or therapeutic agent in the lipid particle is not significantly degraded after exposure to serum or a nuclease or

protease assay that would significantly degrade free DNA, RNA, or protein. In a fully encapsulated system, preferably less than about 25% of the active agent or therapeutic agent in the particle is degraded in a treatment that would normally degrade 100% of free active agent or therapeutic agent, more preferably less than about 10%, and most preferably less than about 5% of the active agent or therapeutic agent in the particle is degraded. In the context of nucleic acid therapeutic agents, full encapsulation may be determined by an Oligreen® assay. Oligreen® is an ultra-sensitive fluorescent nucleic acid stain for quantitating oligonucleotides and single-stranded DNA or RNA in solution (available from Invitrogen Corporation; Carlsbad, CA). "Fully encapsulated" also indicates that the lipid particles are serum-stable, that is, that they do not rapidly decompose into their component parts upon *in vivo* administration.

[0142] In another aspect, the present invention provides a lipid particle (*e.g.*, SNALP) composition comprising a plurality of lipid particles. In preferred embodiments, the active agent or therapeutic agent (*e.g.*, nucleic acid) is fully encapsulated within the lipid portion of the lipid particles (*e.g.*, SNALP), such that from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, %, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% (or any fraction thereof or range therein) of the lipid particles (*e.g.*, SNALP) have the active agent or therapeutic agent encapsulated therein.

[0143] Typically, the lipid particles (*e.g.*, SNALP) of the invention have a lipid:active agent (*e.g.*, lipid:nucleic acid) ratio (mass/mass ratio) of from about 1 to about 100. In some instances, the lipid:active agent (*e.g.*, lipid:nucleic acid) ratio (mass/mass ratio) ranges from about 1 to about 50, from about 2 to about 25, from about 3 to about 20, from about 4 to about 15, or from about 5 to about 10. In preferred embodiments, the lipid particles of the invention have a lipid:active agent (*e.g.*, lipid:nucleic acid) ratio (mass/mass ratio) of from about 5 to about 15, *e.g.*, about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 (or any fraction thereof or range therein).

[0144] Typically, the lipid particles (*e.g.*, SNALP) of the invention have a mean diameter of from about 40 nm to about 150 nm. In preferred embodiments, the lipid particles (*e.g.*, SNALP) of the invention have a mean diameter of from about 40 nm to about 130 nm, from about 40 nm to about 120 nm, from about 40 nm to about 100 nm, from about 50 nm to about 120 nm, from about 50 nm to about 100 nm, from about 60 nm to about 120 nm, from about 60 nm to about 110 nm, from about 60 nm to about 100 nm, from about 60 nm to about 90 nm, from about 60 nm to about 80 nm, from about 70 nm to about 120 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 70 nm to about 90 nm, from about 70 nm to about 80 nm, or less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm (or any fraction thereof or range therein).

[0145] In one specific embodiment of the invention, the SNALP comprises: (a) one or more unmodified and/or modified interfering RNA (*e.g.*, siRNA, aiRNA, miRNA) that silence target gene expression; (b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This specific embodiment of SNALP is generally referred to herein as the "1:62" formulation. In a preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is cholesterol, and the conjugated lipid is a PEG-DAA conjugate. Although these are preferred embodiments of the 1:62 formulation, those of skill in the art will appreciate that other cationic lipids, non-cationic lipids (including other cholesterol derivatives), and conjugated lipids can be used in the 1:62 formulation as described herein.

[0146] In another specific embodiment of the invention, the SNALP comprises: (a) one or more unmodified and/or modified interfering RNA (*e.g.*, siRNA, aiRNA, miRNA) that silence target gene expression; (b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This specific embodiment of SNALP is generally referred to herein as the "1:57" formulation. In one preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is a mixture of a phospholipid (such as DPPC) and cholesterol, wherein the phospholipid comprises from about 5 mol % to about 9 mol % of the total lipid present in the particle (*e.g.*,

about 7.1 mol %) and the cholesterol (or cholesterol derivative) comprises from about 32 mol % to about 37 mol % of the total lipid present in the particle (*e.g.*, about 34.3 mol %), and the PEG-lipid is a PEG-DAA (*e.g.*, PEG-cDMA). In another preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is a mixture of a phospholipid (such as DPPC) and cholesterol, wherein the phospholipid comprises from about 15 mol % to about 25 mol % of the total lipid present in the particle (*e.g.*, about 20 mol %) and the cholesterol (or cholesterol derivative) comprises from about 15 mol % to about 25 mol % of the total lipid present in the particle (*e.g.*, about 20 mol %), and the PEG-lipid is a PEG-DAA (*e.g.*, PEG-cDMA). Although these are preferred embodiments of the 1:57 formulation, those of skill in the art will appreciate that other cationic lipids, non-cationic lipids (including other phospholipids and other cholesterol derivatives), and conjugated lipids can be used in the 1:57 formulation as described herein.

[0147] In preferred embodiments, the 1:62 SNALP formulation is a three-component system which is phospholipid-free and comprises about 1.5 mol % PEG-cDMA (or PEG-cDSA), about 61.5 mol % DLinDMA (or XTC2), and about 36.9 mol % cholesterol (or derivative thereof). In other preferred embodiments, the 1:57 SNALP formulation is a four-component system which comprises about 1.4 mol % PEG-cDMA (or PEG-cDSA), about 57.1 mol % DLinDMA (or XTC2), about 7.1 mol % DPPC, and about 34.3 mol % cholesterol (or derivative thereof). In yet other preferred embodiments, the 1:57 SNALP formulation is a four-component system which comprises about 1.4 mol % PEG-cDMA (or PEG-cDSA), about 57.1 mol % DLinDMA (or XTC2), about 20 mol % DPPC, and about 20 mol % cholesterol (or derivative thereof). It should be understood that these SNALP formulations are target formulations, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the SNALP formulations may vary.

[0148] The present invention also provides a pharmaceutical composition comprising a lipid particle (*e.g.*, SNALP) described herein and a pharmaceutically acceptable carrier.

[0149] In a further aspect, the present invention provides a method for introducing one or more active agents or therapeutic agents (*e.g.*, nucleic acid) into a cell, comprising contacting the cell with a lipid particle (*e.g.*, SNALP) described herein. In one embodiment, the cell is in a mammal and the mammal is a human. In another embodiment, the present invention provides a method for the *in vivo* delivery of one or more active agents or therapeutic agents (*e.g.*, nucleic acid), comprising administering to a mammalian subject a lipid particle (*e.g.*, SNALP) described herein. In a preferred embodiment, the mode of administration includes,

but is not limited to, oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal. Preferably, the mammalian subject is a human.

[0150] In one embodiment, at least about 5%, 10%, 15%, 20%, or 25% of the total injected dose of the lipid particles (*e.g.,* SNALP) is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In other embodiments, more than about 20%, 30%, 40% and as much as about 60%, 70% or 80% of the total injected dose of the lipid particles (*e.g.,* SNALP) is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In certain instances, more than about 10% of a plurality of the particles is present in the plasma of a mammal about 1 hour after administration. In certain other instances, the presence of the lipid particles (*e.g.,* SNALP) is detectable at least about 1 hour after administration of the particle. In certain embodiments, the presence of an active agent or therapeutic agent such as an interfering RNA (*e.g.,* siRNA) is detectable in cells of the lung, liver, tumor, or at a site of inflammation at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In other embodiments, downregulation of expression of a target sequence by an active agent or therapeutic agent such as an interfering RNA (*e.g.,* siRNA) is detectable at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In yet other embodiments, downregulation of expression of a target sequence by an active agent or therapeutic agent such as an interfering RNA (*e.g.,* siRNA) occurs preferentially in tumor cells or in cells at a site of inflammation. In further embodiments, the presence or effect of an active agent or therapeutic agent such as an interfering RNA (*e.g.,* siRNA) in cells at a site proximal or distal to the site of administration or in cells of the lung, liver, or a tumor is detectable at about 12, 24, 48, 72, or 96 hours, or at about 6, 8, 10, 12, 14, 16, 18, 19, 20, 22, 24, 26, or 28 days after administration. In additional embodiments, the lipid particles (*e.g.,* SNALP) of the invention are administered parenterally or intraperitoneally.

[0151] In some embodiments, the lipid particles (*e.g.,* SNALP) of the invention are particularly useful in methods for the therapeutic delivery of one or more nucleic acids comprising an interfering RNA sequence (*e.g.,* siRNA). In particular, it is an object of this invention to provide *in vitro* and *in vivo* methods for treatment of a disease or disorder in a mammal (*e.g.,* a rodent such as a mouse or a primate such as a human, chimpanzee, or monkey) by downregulating or silencing the transcription and/or translation of one or more target nucleic acid sequences or genes of interest. As a non-limiting example, the methods of the invention are useful for *in vivo* delivery of interfering RNA (*e.g.,* siRNA) to the liver and/or tumor of a mammalian subject. In certain embodiments, the disease or disorder is

associated with expression and/or overexpression of a gene and expression or overexpression of the gene is reduced by the interfering RNA (*e.g.*, siRNA). In certain other embodiments, a therapeutically effective amount of the lipid particle (*e.g.*, SNALP) may be administered to the mammal. In some instances, an interfering RNA (*e.g.*, siRNA) is formulated into a SNALP, and the particles are administered to patients requiring such treatment. In other instances, cells are removed from a patient, the interfering RNA (*e.g.*, siRNA) is delivered *in vitro* (*e.g.*, using a SNALP described herein), and the cells are reinjected into the patient.

[0152] In an additional aspect, the present invention provides lipid particles (*e.g.*, SNALP) comprising asymmetrical interfering RNA (aiRNA) molecules that silence the expression of a target gene and methods of using such particles to silence target gene expression.

[0153] In one embodiment, the aiRNA molecule comprises a double-stranded (duplex) region of about 10 to about 25 (base paired) nucleotides in length, wherein the aiRNA molecule comprises an antisense strand comprising 5' and 3' overhangs, and wherein the aiRNA molecule is capable of silencing target gene expression.

[0154] In certain instances, the aiRNA molecule comprises a double-stranded (duplex) region of about 12-20, 12-19, 12-18, 13-17, or 14-17 (base paired) nucleotides in length, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 (base paired) nucleotides in length. In certain other instances, the 5' and 3' overhangs on the antisense strand comprise sequences that are complementary to the target RNA sequence, and may optionally further comprise nontargeting sequences. In some embodiments, each of the 5' and 3' overhangs on the antisense strand comprises or consists of one, two, three, four, five, six, seven, or more nucleotides.

[0155] In other embodiments, the aiRNA molecule comprises modified nucleotides selected from the group consisting of 2'OMe nucleotides, 2'F nucleotides, 2'-deoxy nucleotides, 2'-O-MOE nucleotides, LNA nucleotides, and mixtures thereof. In a preferred embodiment, the aiRNA molecule comprises 2'OMe nucleotides. As a non-limiting example, the 2'OMe nucleotides may be selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, and mixtures thereof.

[0156] In a related aspect, the present invention provides lipid particles (*e.g.*, SNALP) comprising microRNA (miRNA) molecules that silence the expression of a target gene and methods of using such compositions to silence target gene expression.

[0157] In one embodiment, the miRNA molecule comprises about 15 to about 60 nucleotides in length, wherein the miRNA molecule is capable of silencing target gene expression.

[0158] In certain instances, the miRNA molecule comprises about 15-50, 15-40, or 15-30 nucleotides in length, more typically about 15-25 or 19-25 nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 nucleotides in length. In a preferred embodiment, the miRNA molecule is a mature miRNA molecule targeting an RNA sequence of interest.

[0159] In some embodiments, the miRNA molecule comprises modified nucleotides selected from the group consisting of 2'OMe nucleotides, 2'F nucleotides, 2'-deoxy nucleotides, 2'-O-MOE nucleotides, LNA nucleotides, and mixtures thereof. In a preferred embodiment, the miRNA molecule comprises 2'OMe nucleotides. As a non-limiting example, the 2'OMe nucleotides may be selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, and mixtures thereof.

[0160] As such, the lipid particles of the invention (*e.g.*, SNALP) are advantageous and suitable for use in the administration of active agents or therapeutic agents such as nucleic acid (*e.g.*, interfering RNA such as siRNA, aiRNA, and/or miRNA) to a subject (*e.g.*, a mammal such as a human) because they are stable in circulation, of a size required for pharmacodynamic behavior resulting in access to extravascular sites, and are capable of reaching target cell populations.

IV. Active Agents

[0161] Active agents (*e.g.*, therapeutic agents) include any molecule or compound capable of exerting a desired effect on a cell, tissue, organ, or subject. Such effects may be, *e.g.*, biological, physiological, and/or cosmetic. Active agents may be any type of molecule or compound including, but not limited to, nucleic acids, peptides, polypeptides, small molecules, and mixtures thereof. Non-limiting examples of nucleic acids include interfering RNA molecules (*e.g.*, siRNA, aiRNA, miRNA), antisense oligonucleotides, plasmids, ribozymes, immunostimulatory oligonucleotides, and mixtures thereof. Examples of peptides or polypeptides include, without limitation, antibodies (*e.g.*, polyclonal antibodies, monoclonal antibodies, antibody fragments; humanized antibodies, recombinant antibodies, recombinant human antibodies, Primatized™ antibodies), cytokines, growth factors, apoptotic factors, differentiation-inducing factors, cell-surface receptors and their ligands, hormones, and mixtures thereof. Examples of small molecules include, but are not limited to, small organic molecules or compounds such as any conventional agent or drug known to those of skill in the art.

[0162] In some embodiments, the active agent is a therapeutic agent, or a salt or derivative thereof. Therapeutic agent derivatives may be therapeutically active themselves or they may

be prodrugs, which become active upon further modification. Thus, in one embodiment, a therapeutic agent derivative retains some or all of the therapeutic activity as compared to the unmodified agent, while in another embodiment, a therapeutic agent derivative is a prodrug that lacks therapeutic activity, but becomes active upon further modification.

A. Nucleic Acids

[0163] In certain embodiments, lipid particles of the present invention are associated with a nucleic acid, resulting in a nucleic acid-lipid particle (*e.g.*, SNALP). In some embodiments, the nucleic acid is fully encapsulated in the lipid particle. As used herein, the term "nucleic acid" includes any oligonucleotide or polynucleotide, with fragments containing up to 60 nucleotides generally termed oligonucleotides, and longer fragments termed polynucleotides. In particular embodiments, oligonucletoides of the invention are from about 15 to about 60 nucleotides in length. Nucleic acid may be administered alone in the lipid particles of the invention, or in combination (*e.g.*, co-administered) with lipid particles of the invention comprising peptides, polypeptides, or small molecules such as conventional drugs.

[0164] In the context of this invention, the terms "polynucleotide" and "oligonucleotide" refer to a polymer or oligomer of nucleotide or nucleoside monomers consisting of naturally-occurring bases, sugars and intersugar (backbone) linkages. The terms "polynucleotide" and "oligonucleotide" also include polymers or oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake, reduced immunogenicity, and increased stability in the presence of nucleases.

[0165] Oligonucleotides are generally classified as deoxyribooligonucleotides or ribooligonucleotides. A deoxyribooligonucleotide consists of a 5-carbon sugar called deoxyribose joined covalently to phosphate at the 5′ and 3′ carbons of this sugar to form an alternating, unbranched polymer. A ribooligonucleotide consists of a similar repeating structure where the 5-carbon sugar is ribose.

[0166] The nucleic acid that is present in a lipid-nucleic acid particle according to this invention includes any form of nucleic acid that is known. The nucleic acids used herein can be single-stranded DNA or RNA, or double-stranded DNA or RNA, or DNA-RNA hybrids. Examples of double-stranded DNA are described herein and include, *e.g.*, structural genes, genes including control and termination regions, and self-replicating systems such as viral or plasmid DNA. Examples of double-stranded RNA are described herein and include, *e.g.*,

siRNA and other RNAi agents such as aiRNA and pre-miRNA. Single-stranded nucleic acids include, *e.g.*, antisense oligonucleotides, ribozymes, mature miRNA, and triplex-forming oligonucleotides.

[0167] Nucleic acids of the invention may be of various lengths, generally dependent upon the particular form of nucleic acid. For example, in particular embodiments, plasmids or genes may be from about 1,000 to about 100,000 nucleotide residues in length. In particular embodiments, oligonucleotides may range from about 10 to about 100 nucleotides in length. In various related embodiments, oligonucleotides, both single-stranded, double-stranded, and triple-stranded, may range in length from about 10 to about 60 nucleotides, from about 15 to about 60 nucleotides, from about 20 to about 50 nucleotides, from about 15 to about 30 nucleotides, or from about 20 to about 30 nucleotides in length.

[0168] In particular embodiments, an oligonucleotide (or a strand thereof) of the invention specifically hybridizes to or is complementary to a target polynucleotide sequence. The terms "specifically hybridizable" and "complementary" as used herein indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. In preferred embodiments, an oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target sequence interferes with the normal function of the target sequence to cause a loss of utility or expression therefrom, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or, in the case of *in vitro* assays, under conditions in which the assays are conducted. Thus, the oligonucleotide may include 1, 2, 3, or more base substitutions as compared to the region of a gene or mRNA sequence that it is targeting or to which it specifically hybridizes.

1. siRNA

[0169] The siRNA component of the nucleic acid-lipid particles of the present invention is capable of silencing the expression of a target gene of interest. Each strand of the siRNA duplex is typically about 15 to about 60 nucleotides in length, preferably about 15 to about 30 nucleotides in length. In certain embodiments, the siRNA comprises at least one modified nucleotide. The modified siRNA is generally less immunostimulatory than a corresponding unmodified siRNA sequence and retains RNAi activity against the target gene of interest. In

some embodiments, the modified siRNA contains at least one 2'OMe purine or pyrimidine nucleotide such as a 2'OMe-guanosine, 2'OMe-uridine, 2'OMe-adenosine, and/or 2'OMe-cytosine nucleotide. In preferred embodiments, one or more of the uridine and/or guanosine nucleotides are modified. The modified nucleotides can be present in one strand (*i.e.*, sense or antisense) or both strands of the siRNA. The siRNA sequences may have overhangs (*e.g.*, 3' or 5' overhangs as described in Elbashir *et al.*, *Genes Dev.*, 15:188 (2001) or Nykänen *et al.*, *Cell,* 107:309 (2001)), or may lack overhangs (*i.e.*, have blunt ends).

[0170] The modified siRNA generally comprises from about 1% to about 100% (*e.g.*, about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) modified nucleotides in the double-stranded region of the siRNA duplex. In certain embodiments, one, two, three, four, five, six, seven, eight, nine, ten, or more of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0171] In some embodiments, less than about 25% (*e.g.*, less than about 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1%) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0172] In other embodiments, from about 1% to about 25% (*e.g.*, from about 1%-25%, 2%-25%, 3%-25%, 4%-25%, 5%-25%, 6%-25%, 7%-25%, 8%-25%, 9%-25%, 10%-25%, 11%-25%, 12%-25%, 13%-25%, 14%-25%, 15%-25%, 16%-25%, 17%-25%, 18%-25%, 19%-25%, 20%-25%, 21%-25%, 22%-25%, 23%-25%, 24%-25%, *etc.*) or from about 1% to about 20% (*e.g.*, from about 1%-20%, 2%-20%, 3%-20%, 4%-20%, 5%-20%, 6%-20%, 7%-20%, 8%-20%, 9%-20%, 10%-20%, 11%-20%, 12%-20%, 13%-20%, 14%-20%, 15%-20%, 16%-20%, 17%-20%, 18%-20%, 19%-20%, 1%-19%, 2%-19%, 3%-19%, 4%-19%, 5%-19%, 6%-19%, 7%-19%, 8%-19%, 9%-19%, 10%-19%, 11%-19%, 12%-19%, 13%-19%, 14%-19%, 15%-19%, 16%-19%, 17%-19%, 18%-19%, 1%-18%, 2%-18%, 3%-18%, 4%-18%, 5%-18%, 6%-18%, 7%-18%, 8%-18%, 9%-18%, 10%-18%, 11%-18%, 12%-18%, 13%-18%, 14%-18%, 15%-18%, 16%-18%, 17%-18%, 1%-17%, 2%-17%, 3%-17%, 4%-17%, 5%-17%, 6%-17%, 7%-17%, 8%-17%, 9%-17%, 10%-17%, 11%-17%, 12%-17%, 13%-17%, 14%-17%, 15%-17%, 16%-17%, 1%-16%, 2%-16%, 3%-16%, 4%-16%, 5%-16%, 6%-16%, 7%-16%, 8%-16%, 9%-16%, 10%-16%, 11%-16%, 12%-16%, 13%-16%, 14%-16%, 15%-16%, 1%-15%, 2%-15%, 3%-15%, 4%-15%, 5%-15%, 6%-15%, 7%-15%, 8%-15%, 9%-

15%, 10%-15%, 11%-15%, 12%-15%, 13%-15%, 14%-15%, *etc.*) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0173] In further embodiments, *e.g.*, when one or both strands of the siRNA are selectively modified at uridine and/or guanosine nucleotides, the resulting modified siRNA can comprise less than about 30% modified nucleotides (*e.g.*, less than about 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% modified nucleotides) or from about 1% to about 30% modified nucleotides (*e.g.*, from about 1%-30%, 2%-30%, 3%-30%, 4%-30%, 5%-30%, 6%-30%, 7%-30%, 8%-30%, 9%-30%, 10%-30%, 11%-30%, 12%-30%, 13%-30%, 14%-30%, 15%-30%, 16%-30%, 17%-30%, 18%-30%, 19%-30%, 20%-30%, 21%-30%, 22%-30%, 23%-30%, 24%-30%, 25%-30%, 26%-30%, 27%-30%, 28%-30%, or 29%-30% modified nucleotides).

a. Selection of siRNA Sequences

[0174] Suitable siRNA sequences can be identified using any means known in the art. Typically, the methods described in Elbashir *et al.*, *Nature*, 411:494-498 (2001) and Elbashir *et al.*, *EMBO J.*, 20:6877-6888 (2001) are combined with rational design rules set forth in Reynolds *et al.*, *Nature Biotech.*, 22(3):326-330 (2004).

[0175] Generally, the nucleotide sequence 3' of the AUG start codon of a transcript from the target gene of interest is scanned for dinucleotide sequences (*e.g.*, AA, NA, CC, GG, or UU, wherein N = C, G, or U) (*see*, *e.g.*, Elbashir *et al.*, *EMBO J.*, 20:6877-6888 (2001)). The nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences (*i.e.*, a target sequence or a sense strand sequence). Typically, the 19, 21, 23, 25, 27, 29, 31, 33, 35, or more nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences. In some embodiments, the dinucleotide sequence is an AA or NA sequence and the 19 nucleotides immediately 3' to the AA or NA dinucleotide are identified as potential siRNA sequences. siRNA sequences are usually spaced at different positions along the length of the target gene. To further enhance silencing efficiency of the siRNA sequences, potential siRNA sequences may be analyzed to identify sites that do not contain regions of homology to other coding sequences, *e.g.*, in the target cell or organism. For example, a suitable siRNA sequence of about 21 base pairs typically will not have more than 16-17 contiguous base pairs of homology to coding sequences in the target cell or organism. If the siRNA sequences are to be expressed from an RNA Pol III promoter, siRNA sequences lacking more than 4 contiguous A's or T's are selected.

[0176] Once a potential siRNA sequence has been identified, a complementary sequence (*i.e.*, an antisense strand sequence) can be designed. A potential siRNA sequence can also be analyzed using a variety of criteria known in the art. For example, to enhance their silencing efficiency, the siRNA sequences may be analyzed by a rational design algorithm to identify sequences that have one or more of the following features: (1) G/C content of about 25% to about 60% G/C; (2) at least 3 A/Us at positions 15-19 of the sense strand; (3) no internal repeats; (4) an A at position 19 of the sense strand; (5) an A at position 3 of the sense strand; (6) a U at position 10 of the sense strand; (7) no G/C at position 19 of the sense strand; and (8) no G at position 13 of the sense strand. siRNA design tools that incorporate algorithms that assign suitable values of each of these features and are useful for selection of siRNA can be found at, *e.g.*, http://boz094.ust.hk/RNAi/siRNA. One of skill in the art will appreciate that sequences with one or more of the foregoing characteristics may be selected for further analysis and testing as potential siRNA sequences.

[0177] Additionally, potential siRNA sequences with one or more of the following criteria can often be eliminated as siRNA: (1) sequences comprising a stretch of 4 or more of the same base in a row; (2) sequences comprising homopolymers of Gs (*i.e.*, to reduce possible non-specific effects due to structural characteristics of these polymers; (3) sequences comprising triple base motifs (*e.g.*, GGG, CCC, AAA, or TTT); (4) sequences comprising stretches of 7 or more G/Cs in a row; and (5) sequences comprising direct repeats of 4 or more bases within the candidates resulting in internal fold-back structures. However, one of skill in the art will appreciate that sequences with one or more of the foregoing characteristics may still be selected for further analysis and testing as potential siRNA sequences.

[0178] In some embodiments, potential siRNA sequences may be further analyzed based on siRNA duplex asymmetry as described in, *e.g.*, Khvorova *et al.*, *Cell*, 115:209-216 (2003); and Schwarz *et al.*, *Cell*, 115:199-208 (2003). In other embodiments, potential siRNA sequences may be further analyzed based on secondary structure at the target site as described in, *e.g.*, Luo *et al.*, *Biophys. Res. Commun.*, 318:303-310 (2004). For example, secondary structure at the target site can be modeled using the Mfold algorithm (available at http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi) to select siRNA sequences which favor accessibility at the target site where less secondary structure in the form of base-pairing and stem-loops is present.

[0179] Once a potential siRNA sequence has been identified, the sequence can be analyzed for the presence of any immunostimulatory properties, *e.g.*, using an *in vitro* cytokine assay or an *in vivo* animal model. Motifs in the sense and/or antisense strand of the siRNA

sequence such as GU-rich motifs (*e.g.*, 5'-GU-3', 5'-UGU-3', 5'-GUGU-3', 5'-UGUGU-3', *etc.*) can also provide an indication of whether the sequence may be immunostimulatory. Once an siRNA molecule is found to be immunostimulatory, it can then be modified to decrease its immunostimulatory properties as described herein. As a non-limiting example, an siRNA sequence can be contacted with a mammalian responder cell under conditions such that the cell produces a detectable immune response to determine whether the siRNA is an immunostimulatory or a non-immunostimulatory siRNA. The mammalian responder cell may be from a naïve mammal (*i.e.*, a mammal that has not previously been in contact with the gene product of the siRNA sequence). The mammalian responder cell may be, *e.g.*, a peripheral blood mononuclear cell (PBMC), a macrophage, and the like. The detectable immune response may comprise production of a cytokine or growth factor such as, *e.g.*, TNF-α, IFN-α, IFN-β, IFN-γ, IL-6, IL-12, or a combination thereof. An siRNA molecule identified as being immunostimulatory can then be modified to decrease its immunostimulatory properties by replacing at least one of the nucleotides on the sense and/or antisense strand with modified nucleotides. For example, less than about 30% (*e.g.*, less than about 30%, 25%, 20%, 15%, 10%, or 5%) of the nucleotides in the double-stranded region of the siRNA duplex can be replaced with modified nucleotides such as 2'OMe nucleotides. The modified siRNA can then be contacted with a mammalian responder cell as described above to confirm that its immunostimulatory properties have been reduced or abrogated.

[0180] Suitable *in vitro* assays for detecting an immune response include, but are not limited to, the double monoclonal antibody sandwich immunoassay technique of David *et al.* (U.S. Patent No. 4,376,110); monoclonal-polyclonal antibody sandwich assays (Wide *et al.*, in Kirkham and Hunter, eds., *Radioimmunoassay Methods*, E. and S. Livingstone, Edinburgh (1970)); the "Western blot" method of Gordon *et al.* (U.S. Patent No. 4,452,901); immunoprecipitation of labeled ligand (Brown *et al.*, *J. Biol. Chem.*, 255:4980-4983 (1980)); enzyme-linked immunosorbent assays (ELISA) as described, for example, by Raines *et al.*, *J. Biol. Chem.*, 257:5154-5160 (1982); immunocytochemical techniques, including the use of fluorochromes (Brooks *et al.*, *Clin. Exp. Immunol.*, 39:477 (1980)); and neutralization of activity (Bowen-Pope *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:2396-2400 (1984)). In addition to the immunoassays described above, a number of other immunoassays are available, including those described in U.S. Patent Nos. 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0181] A non-limiting example of an *in vivo* model for detecting an immune response includes an *in vivo* mouse cytokine induction assay as described in, *e.g.,* Judge *et al., Mol. Ther.*, 13:494-505 (2006). In certain embodiments, the assay that can be performed as follows: (1) siRNA can be administered by standard intravenous injection in the lateral tail vein; (2) blood can be collected by cardiac puncture about 6 hours after administration and processed as plasma for cytokine analysis; and (3) cytokines can be quantified using sandwich ELISA kits according to the manufacturer's instructions (*e.g.,* mouse and human IFN-α (PBL Biomedical; Piscataway, NJ); human IL-6 and TNF-α (eBioscience; San Diego, CA); and mouse IL-6, TNF-α, and IFN-γ (BD Biosciences; San Diego, CA)).

[0182] Monoclonal antibodies that specifically bind cytokines and growth factors are commercially available from multiple sources and can be generated using methods known in the art (*see, e.g.*, Kohler *et al.*, *Nature*, 256: 495-497 (1975) and Harlow and Lane, ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publication, New York (1999)). Generation of monoclonal antibodies has been previously described and can be accomplished by any means known in the art (Buhring *et al.*, in Hybridoma, Vol. 10, No. 1, pp. 77-78 (1991)). In some methods, the monoclonal antibody is labeled (*e.g.*, with any composition detectable by spectroscopic, photochemical, biochemical, electrical, optical, or chemical means) to facilitate detection.

b. Generating siRNA Molecules

[0183] siRNA can be provided in several forms including, *e.g.*, as one or more isolated small-interfering RNA (siRNA) duplexes, as longer double-stranded RNA (dsRNA), or as siRNA or dsRNA transcribed from a transcriptional cassette in a DNA plasmid. The siRNA sequences may have overhangs (*e.g.*, 3' or 5' overhangs as described in Elbashir *et al.*, *Genes Dev.*, 15:188 (2001) or Nykänen *et al.*, *Cell*, 107:309 (2001), or may lack overhangs (*i.e.*, to have blunt ends).

[0184] An RNA population can be used to provide long precursor RNAs, or long precursor RNAs that have substantial or complete identity to a selected target sequence can be used to make the siRNA. The RNAs can be isolated from cells or tissue, synthesized, and/or cloned according to methods well known to those of skill in the art. The RNA can be a mixed population (obtained from cells or tissue, transcribed from cDNA, subtracted, selected, *etc.*), or can represent a single target sequence. RNA can be naturally occurring (*e.g.*, isolated from tissue or cell samples), synthesized *in vitro (e.g.,* using T7 or SP6 polymerase and PCR products or a cloned cDNA), or chemically synthesized.

[0185] To form a long dsRNA, for synthetic RNAs, the complement is also transcribed *in vitro* and hybridized to form a dsRNA. If a naturally occuring RNA population is used, the RNA complements are also provided (*e.g.*, to form dsRNA for digestion by *E. coli* RNAse III or Dicer), *e.g.*, by transcribing cDNAs corresponding to the RNA population, or by using RNA polymerases. The precursor RNAs are then hybridized to form double stranded RNAs for digestion. The dsRNAs can be directly administered to a subject or can be digested *in vitro* prior to administration.

[0186] Methods for isolating RNA, synthesizing RNA, hybridizing nucleic acids, making and screening cDNA libraries, and performing PCR are well known in the art (*see, e.g.,* Gubler and Hoffman, *Gene*, 25:263-269 (1983); Sambrook *et al., supra*; Ausubel *et al., supra*), as are PCR methods (*see,* U.S. Patent Nos. 4,683,195 and 4,683,202; *PCR Protocols: A Guide to Methods and Applications* (Innis *et al.*, eds, 1990)). Expression libraries are also well known to those of skill in the art. Additional basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994). The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0187] Preferably, siRNA are chemically synthesized. The oligonucleotides that comprise the siRNA molecules of the invention can be synthesized using any of a variety of techniques known in the art, such as those described in Usman *et al.*, *J. Am. Chem. Soc.*, 109:7845 (1987); Scaringe *et al.*, *Nucl. Acids Res.*, 18:5433 (1990); Wincott *et al.*, *Nucl. Acids Res.*, 23:2677-2684 (1995); and Wincott *et al., Methods Mol. Bio.*, 74:59 (1997). The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end and phosphoramidites at the 3'-end. As a non-limiting example, small scale syntheses can be conducted on an Applied Biosystems synthesizer using a 0.2 μmol scale protocol. Alternatively, syntheses at the 0.2 μmol scale can be performed on a 96-well plate synthesizer from Protogene (Palo Alto, CA). However, a larger or smaller scale of synthesis is also within the scope of this invention. Suitable reagents for oligonucleotide synthesis, methods for RNA deprotection, and methods for RNA purification are known to those of skill in the art.

[0188] siRNA molecules can also be synthesized via a tandem synthesis technique, wherein both strands are synthesized as a single continuous oligonucleotide fragment or strand separated by a cleavable linker that is subsequently cleaved to provide separate fragments or

strands that hybridize to form the siRNA duplex. The linker can be a polynucleotide linker or a non-nucleotide linker. The tandem synthesis of siRNA can be readily adapted to both multiwell/multiplate synthesis platforms as well as large scale synthesis platforms employing batch reactors, synthesis columns, and the like. Alternatively, siRNA molecules can be assembled from two distinct oligonucleotides, wherein one oligonucleotide comprises the sense strand and the other comprises the antisense strand of the siRNA. For example, each strand can be synthesized separately and joined together by hybridization or ligation following synthesis and/or deprotection. In certain other instances, siRNA molecules can be synthesized as a single continuous oligonucleotide fragment, where the self-complementary sense and antisense regions hybridize to form an siRNA duplex having hairpin secondary structure.

c. Modifying siRNA Sequences

[0189] In certain aspects, siRNA molecules comprise a duplex having two strands and at least one modified nucleotide in the double-stranded region, wherein each strand is about 15 to about 60 nucleotides in length. Advantageously, the modified siRNA is less immunostimulatory than a corresponding unmodified siRNA sequence, but retains the capability of silencing the expression of a target sequence. In preferred embodiments, the degree of chemical modifications introduced into the siRNA molecule strikes a balance between reduction or abrogation of the immunostimulatory properties of the siRNA and retention of RNAi activity. As a non-limiting example, an siRNA molecule that targets a gene of interest can be minimally modified (*e.g.*, less than about 30%, 25%, 20%, 15%, 10%, or 5% modified) at selective uridine and/or guanosine nucleotides within the siRNA duplex to eliminate the immune response generated by the siRNA while retaining its capability to silence target gene expression.

[0190] Examples of modified nucleotides suitable for use in the invention include, but are not limited to, ribonucleotides having a 2'-O-methyl (2'OMe), 2'-deoxy-2'-fluoro (2'F), 2'-deoxy, 5-C-methyl, 2'-O-(2-methoxyethyl) (MOE), 4'-thio, 2'-amino, or 2'-C-allyl group. Modified nucleotides having a Northern conformation such as those described in, *e.g.*, Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag Ed. (1984), are also suitable for use in siRNA molecules. Such modified nucleotides include, without limitation, locked nucleic acid (LNA) nucleotides (*e.g.*, 2'-O, 4'-C-methylene-(D-ribofuranosyl) nucleotides),

2'-O-(2-methoxyethyl) (MOE) nucleotides, 2'-methyl-thio-ethyl nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy-2'-chloro (2'CI) nucleotides, and 2'-azido nucleotides. In certain instances, the siRNA molecules described herein include one or more G-clamp nucleotides. A G-clamp nucleotide refers to a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine nucleotide within a duplex (*see, e.g.,* Lin *et al., J. Am. Chem. Soc.,* 120:8531-8532 (1998)). In addition, nucleotides having a nucleotide base analog such as, for example, C-phenyl, C-naphthyl, other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole (*see, e.g.,* Loakes, *Nucl. Acids Res.,* 29:2437-2447 (2001)) can be incorporated into siRNA molecules.

[0191] In certain embodiments, siRNA molecules may further comprise one or more chemical modifications such as terminal cap moieties, phosphate backbone modifications, and the like. Examples of terminal cap moieties include, without limitation, inverted deoxy abasic residues, glyceryl modifications, 4',5'-methylene nucleotides, 1-(β-D-erythrofuranosyl) nucleotides, 4'-thio nucleotides, carbocyclic nucleotides, 1,5-anhydrohexitol nucleotides, L-nucleotides, α-nucleotides, modified base nucleotides, *threo*-pentofuranosyl nucleotides, acyclic 3',4'-seco nucleotides, acyclic 3,4-dihydroxybutyl nucleotides, acyclic 3,5-dihydroxypentyl nucleotides, 3'-3'-inverted nucleotide moieties, 3'-3'-inverted abasic moieties, 3'-2'-inverted nucleotide moieties, 3'-2'-inverted abasic moieties, 5'-5'-inverted nucleotide moieties, 5'-5'-inverted abasic moieties, 3'-5'-inverted deoxy abasic moieties, 5'-amino-alkyl phosphate, 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate, 6-aminohexyl phosphate, 1,2-aminododecyl phosphate, hydroxypropyl phosphate, 1,4-butanediol phosphate, 3'-phosphoramidate, 5'-phosphoramidate, hexylphosphate, aminohexyl phosphate, 3'-phosphate, 5'-amino, 3'-phosphorothioate, 5'-phosphorothioate, phosphorodithioate, and bridging or non-bridging methylphosphonate or 5'-mercapto moieties (*see, e.g.,* U.S. Patent No. 5,998,203; Beaucage *et al., Tetrahedron* 49:1925 (1993)). Non-limiting examples of phosphate backbone modifications (*i.e.,* resulting in modified internucleotide linkages) include phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate, carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and alkylsilyl substitutions (*see, e.g.,* Hunziker *et al., Nucleic Acid Analogues: Synthesis and Properties,* in *Modern Synthetic Methods,* VCH, 331-417 (1995);

Mesmaeker *et al.*, *Novel Backbone Replacements for Oligonucleotides*, in *Carbohydrate Modifications in Antisense Research*, ACS, 24-39 (1994)). Such chemical modifications can occur at the 5'-end and/or 3'-end of the sense strand, antisense strand, or both strands of the siRNA. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0192] In some embodiments, the sense and/or antisense strand of the siRNA molecule can further comprise a 3'-terminal overhang having about 1 to about 4 (*e.g.*, 1, 2, 3, or 4) 2'-deoxy ribonucleotides and/or any combination of modified and unmodified nucleotides. Additional examples of modified nucleotides and types of chemical modifications that can be introduced into siRNA molecules are described, *e.g.*, in UK Patent No. GB 2,397,818 B and U.S. Patent Publication Nos. 20040192626, 20050282188, and 20070135372, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0193] The siRNA molecules described herein can optionally comprise one or more non-nucleotides in one or both strands of the siRNA. As used herein, the term "non-nucleotide" refers to any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base such as adenosine, guanine, cytosine, uracil, or thymine and therefore lacks a base at the 1'-position.

[0194] In other embodiments, chemical modification of the siRNA comprises attaching a conjugate to the siRNA molecule. The conjugate can be attached at the 5' and/or 3'-end of the sense and/or antisense strand of the siRNA via a covalent attachment such as, *e.g.*, a biodegradable linker. The conjugate can also be attached to the siRNA, *e.g.*, through a carbamate group or other linking group (*see, e.g.*, U.S. Patent Publication Nos. 20050074771, 20050043219, and 20050158727). In certain instances, the conjugate is a molecule that facilitates the delivery of the siRNA into a cell. Examples of conjugate molecules suitable for attachment to siRNA include, without limitation, steroids such as cholesterol, glycols such as polyethylene glycol (PEG), human serum albumin (HSA), fatty acids, carotenoids, terpenes, bile acids, folates (*e.g.*, folic acid, folate analogs and derivatives thereof), sugars (*e.g.*, galactose, galactosamine, N-acetyl galactosamine, glucose, mannose, fructose, fucose, *etc.*), phospholipids, peptides, ligands for cellular receptors capable of mediating cellular uptake, and combinations thereof (*see, e.g.*, U.S. Patent Publication Nos. 20030130186, 20040110296, and 20040249178; U.S. Patent No. 6,753,423). Other examples include the lipophilic moiety, vitamin, polymer, peptide, protein, nucleic acid, small molecule,

oligosaccharide, carbohydrate cluster, intercalator, minor groove binder, cleaving agent, and cross-linking agent conjugate molecules described in U.S. Patent Publication Nos. 20050119470 and 20050107325. Yet other examples include the 2'-O-alkyl amine, 2'-O-alkoxyalkyl amine, polyamine, C5-cationic modified pyrimidine, cationic peptide, guanidinium group, amidininium group, cationic amino acid conjugate molecules described in U.S. Patent Publication No. 20050153337. Additional examples include the hydrophobic group, membrane active compound, cell penetrating compound, cell targeting signal, interaction modifier, and steric stabilizer conjugate molecules described in U.S. Patent Publication No. 20040167090. Further examples include the conjugate molecules described in U.S. Patent Publication No. 20050239739. The type of conjugate used and the extent of conjugation to the siRNA molecule can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of the siRNA while retaining RNAi activity. As such, one skilled in the art can screen siRNA molecules having various conjugates attached thereto to identify ones having improved properties and full RNAi activity using any of a variety of well-known *in vitro* cell culture or *in vivo* animal models. The disclosures of the above-described patent documents are herein incorporated by reference in their entirety for all purposes.

d. Target Genes

[0195] The siRNA component of the nucleic acid-lipid particles described herein can be used to downregulate or silence the translation (*i.e.*, expression) of a gene of interest. Genes of interest include, but are not limited to, genes associated with viral infection and survival, genes associated with metabolic diseases and disorders (*e.g.*, liver diseases and disorders), genes associated with tumorigenesis and cell transformation (*e.g.*, cancer), angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

[0196] Genes associated with viral infection and survival include those expressed by a virus in order to bind, enter, and replicate in a cell. Of particular interest are viral sequences associated with chronic viral diseases. Viral sequences of particular interest include sequences of Filoviruses such as Ebola virus and Marburg virus (*see, e.g.,* Geisbert *et al., J. Infect. Dis.*, 193:1650-1657 (2006)); Arenaviruses such as Lassa virus, Junin virus, Machupo virus, Guanarito virus, and Sabia virus (Buchmeier *et al., Arenaviridae*: the viruses and their replication, In: FIELDS VIROLOGY, Knipe *et al.* (eds.), 4th ed., Lippincott-Raven, Philadelphia, (2001)); Influenza viruses such as Influenza A, B, and C viruses, (*see, e.g.,*

Steinhauer *et al.*, *Annu Rev Genet.*, 36:305-332 (2002); and Neumann *et al.*, *J Gen Virol.*, 83:2635-2662 (2002)); Hepatitis viruses (*see, e.g.*, Hamasaki *et al.*, *FEBS Lett.*, 543:51 (2003); Yokota *et al.*, *EMBO Rep.*, 4:602 (2003); Schlomai *et al.*, *Hepatology,* 37:764 (2003); Wilson *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:2783 (2003); Kapadia *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:2014 (2003); and FIELDS VIROLOGY, Knipe *et al.* (eds.), 4th ed., Lippincott-Raven, Philadelphia (2001)); Human Immunodeficiency Virus (HIV) (Banerjea *et al.*, *Mol. Ther.*, 8:62 (2003); Song *et al.*, *J. Virol.*, 77:7174 (2003); Stephenson, *JAMA,* 289:1494 (2003); Qin *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:183 (2003)); Herpes viruses (Jia *et al.*, *J. Virol.*, 77:3301 (2003)); and Human Papilloma Viruses (HPV) (Hall *et al.*, *J. Virol.*, 77:6066 (2003); Jiang *et al.*, *Oncogene,* 21:6041 (2002)).

[0197] Exemplary Filovirus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding structural proteins (*e.g.*, VP30, VP35, nucleoprotein (NP), polymerase protein (L-pol)) and membrane-associated proteins (*e.g.*, VP40, glycoprotein (GP), VP24). Complete genome sequences for Ebola virus are set forth in, *e.g.*, Genbank Accession Nos. NC_002549; AY769362; NC_006432; NC_004161; AY729654; AY354458; AY142960; AB050936; AF522874; AF499101; AF272001; and AF086833. Ebola virus VP24 sequences are set forth in, *e.g.*, Genbank Accession Nos. U77385 and AY058897. Ebola virus L-pol sequences are set forth in, *e.g.*, Genbank Accession No. X67110. Ebola virus VP40 sequences are set forth in, *e.g.*, Genbank Accession No. AY058896. Ebola virus NP sequences are set forth in, *e.g.*, Genbank Accession No. AY058895. Ebola virus GP sequences are set forth in, *e.g.*, Genbank Accession No. AY058898; Sanchez *et al.*, *Virus Res.*, 29:215-240 (1993); Will *et al.*, *J. Virol.*, 67:1203-1210 (1993); Volchkov *et al.*, *FEBS Lett.*, 305:181-184 (1992); and U.S. Patent No. 6,713,069. Additional Ebola virus sequences are set forth in, *e.g.*, Genbank Accession Nos. L11365 and X61274. Complete genome sequences for Marburg virus are set forth in, *e.g.*, Genbank Accession Nos. NC_001608; AY430365; AY430366; and AY358025. Marburg virus GP sequences are set forth in, *e.g.*, Genbank Accession Nos. AF005734; AF005733; and AF005732. Marburg virus VP35 sequences are set forth in, *e.g.*, Genbank Accession Nos. AF005731 and AF005730. Additional Marburg virus sequences are set forth in, *e.g.*, Genbank Accession Nos. X64406; Z29337; AF005735; and Z12132. Non-limiting examples of siRNA molecules targeting Ebola virus and Marburg virus nucleic acid sequences include those described in U.S. Patent Publication No. 20070135370, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0198] Exemplary Influenza virus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding nucleoprotein (NP), matrix proteins (M1 and M2), nonstructural proteins (NS1 and NS2), RNA polymerase (PA, PB1, PB2), neuraminidase (NA), and haemagglutinin (HA). Influenza A NP sequences are set forth in, *e.g.*, Genbank Accession Nos. NC_004522; AY818138; AB166863; AB188817; AB189046; AB189054; AB189062; AY646169; AY646177; AY651486; AY651493; AY651494; AY651495; AY651496; AY651497; AY651498; AY651499; AY651500; AY651501; AY651502; AY651503; AY651504; AY651505; AY651506; AY651507; AY651509; AY651528; AY770996; AY790308; AY818138; and AY818140. Influenza A PA sequences are set forth in, *e.g.*, Genbank Accession Nos. AY818132; AY790280; AY646171; AY818132;AY818133; AY646179; AY818134; AY551934; AY651613; AY651610; AY651620; AY651617; AY651600; AY651611; AY651606; AY651618; AY651608; AY651607; AY651605; AY651609; AY651615; AY651616; AY651640; AY651614; AY651612; AY651621; AY651619; AY770995; and AY724786. Non-limiting examples of siRNA molecules targeting Influenza virus nucleic acid sequences include those described in U.S. Patent Publication No. 20070218122, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0199] Exemplary hepatitis virus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences involved in transcription and translation (*e.g.*, En1, En2, X, P) and nucleic acid sequences encoding structural proteins (*e.g.*, core proteins including C and C-related proteins, capsid and envelope proteins including S, M, and/or L proteins, or fragments thereof) (*see*, *e.g.*, FIELDS VIROLOGY, *supra*). Exemplary Hepatits C virus (HCV) nucleic acid sequences that can be silenced include, but are not limited to, the 5'-untranslated region (5'-UTR), the 3'-untranslated region (3'-UTR), the polyprotein translation initiation codon region, the internal ribosome entry site (IRES) sequence, and/or nucleic acid sequences encoding the core protein, the E1 protein, the E2 protein, the p7 protein, the NS2 protein, the NS3 protease/helicase, the NS4A protein, the NS4B protein, the NS5A protein, and/or the NS5B RNA-dependent RNA polymerase. HCV genome sequences are set forth in, *e.g.*, Genbank Accession Nos. NC_004102 (HCV genotype 1a), AJ238799 (HCV genotype 1b), NC_009823 (HCV genotype 2), NC_009824 (HCV genotype 3), NC_009825 (HCV genotype 4), NC_009826 (HCV genotype 5), and NC_009827 (HCV genotype 6). Hepatitis A virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001489; Hepatitis B virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_003977; Hepatitis D virus nucleic acid sequence are set forth in,

e.g., Genbank Accession No. NC_001653; Hepatitis E virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001434; and Hepatitis G virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001710. Silencing of sequences that encode genes associated with viral infection and survival can conveniently be used in combination with the administration of conventional agents used to treat the viral condition. Non-limiting examples of siRNA molecules targeting hepatitis virus nucleic acid sequences include those described in U.S. Patent Publication Nos. 20060281175, 20050058982, and 20070149470; U.S. Patent No. 7,348,314; and U.S. Provisional Application No. 61/162,127, filed March 20, 2009, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0200] Genes associated with metabolic diseases and disorders (*e.g.*, disorders in which the liver is the target and liver diseases and disorders) include, for example, genes expressed in dyslipidemia (*e.g.*, liver X receptors such as LXRα and LXRβ (Genback Accession No. NM_007121), farnesoid X receptors (FXR) (Genbank Accession No. NM_005123), sterol-regulatory element binding protein (SREBP), site-1 protease (S1P), 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG coenzyme-A reductase), apolipoprotein B (ApoB) (Genbank Accession No. NM_000384), apolipoprotein CIII (ApoC3) (Genbank Accession Nos. NM_000040 and NG_008949 REGION: 5001..8164), and apolipoprotein E (ApoE) (Genbank Accession Nos. NM_000041 and NG_007084 REGION: 5001..8612)); and diabetes (*e.g.*, glucose 6-phosphatase) (*see*, *e.g.*, Forman *et al., Cell,* 81:687 (1995); Seol *et al., Mol. Endocrinol.*, 9:72 (1995), Zavacki *et al., Proc. Natl. Acad. Sci. USA*, 94:7909 (1997); Sakai *et al., Cell,* 85:1037-1046 (1996); Duncan *et al., J. Biol. Chem.*, 272:12778-12785 (1997); Willy *et al., Genes Dev.*, 9:1033-1045 (1995); Lehmann *et al., J. Biol. Chem.*, 272:3137-3140 (1997); Janowski *et al., Nature,* 383:728-731 (1996); and Peet *et al., Cell,* 93:693-704 (1998)). One of skill in the art will appreciate that genes associated with metabolic diseases and disorders (*e.g.*, diseases and disorders in which the liver is a target and liver diseases and disorders) include genes that are expressed in the liver itself as well as and genes expressed in other organs and tissues. Silencing of sequences that encode genes associated with metabolic diseases and disorders can conveniently be used in combination with the administration of conventional agents used to treat the disease or disorder. Non-limiting examples of siRNA molecules targeting the ApoB gene include those described in U.S. Patent Publication No. 20060134189, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Non-limiting examples of siRNA molecules

targeting the ApoC3 gene include those described in U.S. Provisional Application No. 61/147,235, filed January 26, 2009, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0201] Examples of gene sequences associated with tumorigenesis and cell transformation (*e.g.*, cancer or other neoplasia) include mitotic kinesins such as Eg5 (KSP, KIF11; Genbank Accession No. NM_004523); serine/threonine kinases such as polo-like kinase 1 (PLK-1) (Genbank Accession No. NM_005030; Barr *et al.*, *Nat. Rev. Mol. Cell Biol.*, 5:429-440 (2004)); tyrosine kinases such as WEE1 (Genbank Accession Nos. NM_003390 and NM_001143976); inhibitors of apoptosis such as XIAP (Genbank Accession No. NM_001167); COP9 signalosome subunits such as CSN1, CSN2, CSN3, CSN4, CSN5 (JAB1; Genbank Accession No. NM_006837); CSN6, CSN7A, CSN7B, and CSN8; ubiquitin ligases such as COP1 (RFWD2; Genbank Accession Nos. NM_022457 and NM_001001740); and histone deacetylases such as HDAC1, HDAC2 (Genbank Accession No. NM_001527), HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, HDAC8, HDAC9, *etc.* Non-limiting examples of siRNA molecules targeting the Eg5 and XIAP genes include those described in U.S. Patent Application No. 11/807,872, filed May 29, 2007, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Non-limiting examples of siRNA molecules targeting the PLK-1 gene include those described in U.S. Patent Publication Nos. 20050107316 and 20070265438; and U.S. Patent Application No. 12/343,342, filed December 23, 2008, the disclosures of which are herein incorporated by reference in their entirety for all purposes. Non-limiting examples of siRNA molecules targeting the CSN5 gene include those described in U.S. Provisional Application No. 61/045,251, filed April 15, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0202] Additional examples of gene sequences associated with tumorigenesis and cell transformation include translocation sequences such as MLL fusion genes, BCR-ABL (Wilda *et al.*, *Oncogene*, 21:5716 (2002); Scherr *et al.*, *Blood*, 101:1566 (2003)), TEL-AML1, EWS-FLI1, TLS-FUS, PAX3-FKHR, BCL-2, AML1-ETO, and AML1-MTG8 (Heidenreich *et al.*, *Blood*, 101:3157 (2003)); overexpressed sequences such as multidrug resistance genes (Nieth *et al.*, *FEBS Lett.*, 545:144 (2003); Wu *et al*, *Cancer Res.* 63:1515 (2003)), cyclins (Li *et al.*, *Cancer Res.*, 63:3593 (2003); Zou *et al.*, *Genes Dev.*, 16:2923 (2002)), beta-catenin (Verma *et al.*, *Clin Cancer Res.*, 9:1291 (2003)), telomerase genes (Kosciolek *et al.*, *Mol Cancer Ther.*, 2:209 (2003)), c-MYC, N-MYC, BCL-2, growth factor receptors (*e.g.*, EGFR/ErbB1 (Genbank Accession Nos. NM_005228, NM_201282, NM_201283, and NM_201284; *see*

also, Nagy *et al. Exp. Cell Res.,* 285:39-49 (2003), ErbB2/HER-2 (Genbank Accession Nos. NM_004448 and NM_001005862), ErbB3 (Genbank Accession Nos. NM_001982 and NM_001005915), and ErbB4 (Genbank Accession Nos. NM_005235 and NM_001042599); and mutated sequences such as RAS (reviewed in Tuschl and Borkhardt, *Mol. Interventions,* 2:158 (2002)). Non-limiting examples of siRNA molecules targeting the EGFR gene include those described in U.S. Patent Application No. 11/807,872, filed May 29, 2007, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0203] Silencing of sequences that encode DNA repair enzymes find use in combination with the administration of chemotherapeutic agents (Collis *et al.*, *Cancer Res.*, 63:1550 (2003)). Genes encoding proteins associated with tumor migration are also target sequences of interest, for example, integrins, selectins, and metalloproteinases. The foregoing examples are not exclusive. Those of skill in the art will understand that any whole or partial gene sequence that facilitates or promotes tumorigenesis or cell transformation, tumor growth, or tumor migration can be included as a template sequence.

[0204] Angiogenic genes are able to promote the formation of new vessels. Of particular interest is vascular endothelial growth factor (VEGF) (Reich *et al.*, *Mol. Vis.*, 9:210 (2003)) or VEGFR. siRNA sequences that target VEGFR are set forth in, *e.g.*, GB 2396864; U.S. Patent Publication No. 20040142895; and CA 2456444, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0205] Anti-angiogenic genes are able to inhibit neovascularization. These genes are particularly useful for treating those cancers in which angiogenesis plays a role in the pathological development of the disease. Examples of anti-angiogenic genes include, but are not limited to, endostatin (*see, e.g.,* U.S. Patent No. 6,174,861), angiostatin (*see, e.g.*, U.S. Patent No. 5,639,725), and VEGFR2 (*see, e.g.,* Decaussin *et al.*, *J. Pathol.*, 188: 369-377 (1999)), the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0206] Immunomodulator genes are genes that modulate one or more immune responses. Examples of immunomodulator genes include, without limitation, cytokines such as growth factors (*e.g.*, TGF-α, TGF-□β, EGF, FGF, IGF, NGF, PDGF, CGF, GM-CSF, SCF, *etc.*), interleukins (*e.g.*, IL-2, IL-4, IL-12 (Hill *et al.*, *J. Immunol.*, 171:691 (2003)), IL-15, IL-18, IL-20, *etc.*), interferons (*e.g.*, IFN-α, IFN-β, IFN-γ, *etc.*) and TNF. Fas and Fas ligand genes are also immunomodulator target sequences of interest (Song *et al.*, *Nat. Med.,* 9:347 (2003)). Genes encoding secondary signaling molecules in hematopoietic and lymphoid cells are also

included in the present invention, for example, Tec family kinases such as Bruton's tyrosine kinase (Btk) (Heinonen *et al.*, *FEBS Lett.*, 527:274 (2002)).

[0207] Cell receptor ligands include ligands that are able to bind to cell surface receptors (*e.g.*, insulin receptor, EPO receptor, G-protein coupled receptors, receptors with tyrosine kinase activity, cytokine receptors, growth factor receptors, *etc.*), to modulate (*e.g.*, inhibit, activate, *etc.*) the physiological pathway that the receptor is involved in (*e.g.*, glucose level modulation, blood cell development, mitogenesis, *etc.*). Examples of cell receptor ligands include, but are not limited to, cytokines, growth factors, interleukins, interferons, erythropoietin (EPO), insulin, glucagon, G-protein coupled receptor ligands, *etc.* Templates coding for an expansion of trinucleotide repeats (*e.g.*, CAG repeats) find use in silencing pathogenic sequences in neurodegenerative disorders caused by the expansion of trinucleotide repeats, such as spinobulbular muscular atrophy and Huntington's Disease (Caplen *et al.*, *Hum. Mol. Genet.*, 11:175 (2002)).

[0208] In addition to its utility in silencing the expression of any of the above-described genes for therapeutic purposes, the siRNA described herein are also useful in research and development applications as well as diagnostic, prophylactic, prognostic, clinical, and other healthcare applications. As a non-limiting example, the siRNA can be used in target validation studies directed at testing whether a gene of interest has the potential to be a therapeutic target. The siRNA can also be used in target identification studies aimed at discovering genes as potential therapeutic targets.

2. aiRNA

[0209] Like siRNA, asymmetrical interfering RNA (aiRNA) can recruit the RNA-induced silencing complex (RISC) and lead to effective silencing of a variety of genes in mammalian cells by mediating sequence-specific cleavage of the target sequence between nucleotide 10 and 11 relative to the 5' end of the antisense strand (Sun *et al.*, *Nat. Biotech.*, 26:1379-1382 (2008)). Typically, an aiRNA molecule comprises a short RNA duplex having a sense strand and an antisense strand, wherein the duplex contains overhangs at the 3' and 5' ends of the antisense strand. The aiRNA is generally asymmetric because the sense strand is shorter on both ends when compared to the complementary antisense strand. In some aspects, aiRNA molecules may be designed, synthesized, and annealed under conditions similar to those used for siRNA molecules. As a non-limiting example, aiRNA sequences may be selected and generated using the methods described above for selecting siRNA sequences.

[0210] In another embodiment, aiRNA duplexes of various lengths (*e.g.*, about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 base pairs, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 base pairs) may be designed with overhangs at the 3' and 5' ends of the antisense strand to target an mRNA of interest. In certain instances, the sense strand of the aiRNA molecule is about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 nucleotides in length, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length. In certain other instances, the antisense strand of the aiRNA molecule is about 15-60, 15-50, or 15-40 nucleotides in length, more typically about 15-30, 15-25, or 19-25 nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 nucleotides in length.

[0211] In some embodiments, the 5' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.*, "AA", "UU", "dTdT", *etc.*). In other embodiments, the 3' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.*, "AA", "UU", "dTdT", *etc.*). In certain aspects, the aiRNA molecules described herein may comprise one or more modified nucleotides, *e.g.*, in the double-stranded (duplex) region and/or in the antisense overhangs. As a non-limiting example, aiRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the aiRNA molecule comprises 2'OMe nucleotides such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0212] In certain embodiments, aiRNA molecules may comprise an antisense strand which corresponds to the antisense strand of an siRNA molecule, *e.g.*, one of the siRNA molecules described herein. In other embodiments, aiRNA molecules may be used to silence the expression of any of the target genes set forth above, such as, *e.g.*, genes associated with viral infection and survival, genes associated with metabolic diseases and disorders, genes associated with tumorigenesis and cell transformation, angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

3. miRNA

[0213] Generally, microRNAs (miRNA) are single-stranded RNA molecules of about 21-23 nucleotides in length which regulate gene expression. miRNAs are encoded by genes from whose DNA they are transcribed, but miRNAs are not translated into protein (non-coding RNA); instead, each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally into a functional mature miRNA. Mature miRNA molecules are either partially or completely complementary to one or more

messenger RNA (mRNA) molecules, and their main function is to downregulate gene expression. The identification of miRNA molecules is described, *e.g.*, in Lagos-Quintana *et al.*, *Science*, 294:853-858; Lau *et al.*, *Science*, 294:858-862; and Lee *et al.*, *Science*, 294:862-864.

[0214] The genes encoding miRNA are much longer than the processed mature miRNA molecule. miRNA are first transcribed as primary transcripts or pri-miRNA with a cap and poly-A tail and processed to short, ~70-nucleotide stem-loop structures known as pre-miRNA in the cell nucleus. This processing is performed in animals by a protein complex known as the Microprocessor complex, consisting of the nuclease Drosha and the double-stranded RNA binding protein Pasha (Denli *et al.*, *Nature*, 432:231-235 (2004)). These pre-miRNA are then processed to mature miRNA in the cytoplasm by interaction with the endonuclease Dicer, which also initiates the formation of the RNA-induced silencing complex (RISC) (Bernstein *et al.*, *Nature*, 409:363-366 (2001). Either the sense strand or antisense strand of DNA can function as templates to give rise to miRNA.

[0215] When Dicer cleaves the pre-miRNA stem-loop, two complementary short RNA molecules are formed, but only one is integrated into the RISC complex. This strand is known as the guide strand and is selected by the argonaute protein, the catalytically active RNase in the RISC complex, on the basis of the stability of the 5' end (Preall *et al.*, *Curr. Biol.*, 16:530-535 (2006)). The remaining strand, known as the anti-guide or passenger strand, is degraded as a RISC complex substrate (Gregory *et al.*, *Cell*, 123:631-640 (2005)). After integration into the active RISC complex, miRNAs base pair with their complementary mRNA molecules and induce target mRNA degradation and/or translational silencing.

[0216] Mammalian miRNA molecules are usually complementary to a site in the 3' UTR of the target mRNA sequence. In certain instances, the annealing of the miRNA to the target mRNA inhibits protein translation by blocking the protein translation machinery. In certain other instances, the annealing of the miRNA to the target mRNA facilitates the cleavage and degradation of the target mRNA through a process similar to RNA interference (RNAi). miRNA may also target methylation of genomic sites which correspond to targeted mRNA. Generally, miRNA function in association with a complement of proteins collectively termed the miRNP.

[0217] In certain aspects, the miRNA molecules described herein are about 15-100, 15-90, 15-80, 15-75, 15-70, 15-60, 15-50, or 15-40 nucleotides in length, more typically about 15-30, 15-25, or 19-25 nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 nucleotides in length. In certain other aspects, miRNA molecules may comprise one or more

modified nucleotides. As a non-limiting example, miRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the miRNA molecule comprises 2'OMe nucleotides such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0218] In some embodiments, miRNA molecules may be used to silence the expression of any of the target genes set forth above, such as, *e.g.*, genes associated with viral infection and survival, genes associated with metabolic diseases and disorders, genes associated with tumorigenesis and cell transformation, angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

[0219] In other embodiments, one or more agents that block the activity of a miRNA targeting an mRNA of interest are administered using a lipid particle of the invention (*e.g.*, a nucleic acid-lipid particle). Examples of blocking agents include, but are not limited to, steric blocking oligonucleotides, locked nucleic acid oligonucleotides, and Morpholino oligonucleotides. Such blocking agents may bind directly to the miRNA or to the miRNA binding site on the target mRNA.

4. Antisense Oligonucleotides

[0220] In one embodiment, the nucleic acid is an antisense oligonucleotide directed to a target gene or sequence of interest. The terms "antisense oligonucleotide" or "antisense" include oligonucleotides that are complementary to a targeted polynucleotide sequence. Antisense oligonucleotides are single strands of DNA or RNA that are complementary to a chosen sequence. Antisense RNA oligonucleotides prevent the translation of complementary RNA strands by binding to the RNA. Antisense DNA oligonucleotides can be used to target a specific, complementary (coding or non-coding) RNA. If binding occurs, this DNA/RNA hybrid can be degraded by the enzyme RNase H. In a particular embodiment, antisense oligonucleotides comprise from about 10 to about 60 nucleotides, more preferably from about 15 to about 30 nucleotides. The term also encompasses antisense oligonucleotides that may not be exactly complementary to the desired target gene. Thus, the invention can be utilized in instances where non-target specific-activities are found with antisense, or where an antisense sequence containing one or more mismatches with the target sequence is the most preferred for a particular use.

[0221] Antisense oligonucleotides have been demonstrated to be effective and targeted inhibitors of protein synthesis, and, consequently, can be used to specifically inhibit protein

synthesis by a targeted gene. The efficacy of antisense oligonucleotides for inhibiting protein synthesis is well established. For example, the synthesis of polygalactauronase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (*see,* U.S. Patent Nos. 5,739,119 and 5,759,829). Furthermore, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDR1), ICAM-1, E-selectin, STK-1, striatal GABAA receptor, and human EGF (*see,* Jaskulski *et al.*, *Science*, 240:1544-6 (1988); Vasanthakumar *et al.*, *Cancer Commun.*, 1:225-32 (1989); Peris *et al.*, *Brain Res Mol Brain Res.*, 15;57:310-20 (1998); and U.S. Patent Nos. 5,801,154; 5,789,573; 5,718,709 and 5,610,288). Moreover, antisense constructs have also been described that inhibit and can be used to treat a variety of abnormal cellular proliferations, *e.g.,* cancer (*see,* U.S. Patent Nos. 5,747,470; 5,591,317; and 5,783,683). The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0222] Methods of producing antisense oligonucleotides are known in the art and can be readily adapted to produce an antisense oligonucleotide that targets any polynucleotide sequence. Selection of antisense oligonucleotide sequences specific for a given target sequence is based upon analysis of the chosen target sequence and determination of secondary structure, T_m, binding energy, and relative stability. Antisense oligonucleotides may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA include those regions at or near the AUG translation initiation codon and those sequences that are substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software (Molecular Biology Insights) and/or the BLASTN 2.0.5 algorithm software (Altschul *et al.*, *Nucleic Acids Res.*, 25:3389-402 (1997)).

5. Ribozymes

[0223] According to another embodiment of the invention, nucleic acid-lipid particles are associated with ribozymes. Ribozymes are RNA-protein complexes having specific catalytic domains that possess endonuclease activity (*see,* Kim *et al.*, *Proc. Natl. Acad. Sci. USA.*, 84:8788-92 (1987); and Forster *et al.*, *Cell*, 49:211-20 (1987)). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (*see,* Cech *et*

al., *Cell*, 27:487-96 (1981); Michel *et al.*, *J. Mol. Biol.*, 216:585-610 (1990); Reinhold-Hurek *et al.*, *Nature*, 357:173-6 (1992)). This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

[0224] At least six basic varieties of naturally-occurring enzymatic RNA molecules are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of an enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

[0225] The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence), or Neurospora VS RNA motif, for example. Specific examples of hammerhead motifs are described in, *e.g.*, Rossi *et al.*, *Nucleic Acids Res.*, 20:4559-65 (1992). Examples of hairpin motifs are described in, *e.g.*, EP 0360257, Hampel *et al.*, *Biochemistry*, 28:4929-33 (1989); Hampel *et al.*, *Nucleic Acids Res.*, 18:299-304 (1990); and U.S. Patent No. 5,631,359. An example of the hepatitis δ virus motif is described in, *e.g.*, Perrotta *et al.*, *Biochemistry*, 31:11843-52 (1992). An example of the RNaseP motif is described in, *e.g.*, Guerrier-Takada *et al.*, *Cell*, 35:849-57 (1983). Examples of the Neurospora VS RNA ribozyme motif is described in, *e.g.*, Saville *et al.*, *Cell*, 61:685-96 (1990); Saville *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:8826-30 (1991); Collins *et al.*, *Biochemistry*, 32:2795-9 (1993). An example of the Group I intron is described in, *e.g.*, U.S. Patent No. 4,987,071. Important characteristics of enzymatic nucleic acid molecules used according to the invention are that they have a specific substrate binding site which is complementary to one or more of the target gene DNA or RNA regions, and that they have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus, the ribozyme constructs need not be limited to specific motifs mentioned herein. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0226] Methods of producing a ribozyme targeted to any polynucleotide sequence are known in the art. Ribozymes may be designed as described in, *e.g.*, PCT Publication Nos. WO 93/23569 and WO 94/02595, and synthesized to be tested *in vitro* and/or *in vivo* as described therein. The disclosures of these PCT publications are herein incorporated by reference in their entirety for all purposes.

[0227] Ribozyme activity can be optimized by altering the length of the ribozyme binding arms or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (*see, e.g.*, PCT Publication Nos. WO 92/07065, WO 93/15187, WO 91/03162, and WO 94/13688; EP 92110298.4; and U.S. Patent No. 5,334,711, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules, the disclosures of which are each herein incorporated by reference in their entirety for all purposes), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

6. Immunostimulatory Oligonucleotides

[0228] Nucleic acids associated with lipid paticles of the present invention may be immunostimulatory, including immunostimulatory oligonucleotides (ISS; single-or double-stranded) capable of inducing an immune response when administered to a subject, which may be a mammal such as a human. ISS include, *e.g.*, certain palindromes leading to hairpin secondary structures (*see,* Yamamoto *et al.*, *J. Immunol.*, 148:4072-6 (1992)), or CpG motifs, as well as other known ISS features (such as multi-G domains; *see;* PCT Publication No. WO 96/11266, the disclosure of which is herein incorporated by reference in its entirety for all purposes).

[0229] Immunostimulatory nucleic acids are considered to be non-sequence specific when it is not required that they specifically bind to and reduce the expression of a target sequence in order to provoke an immune response. Thus, certain immunostimulatory nucleic acids may comprise a sequence corresponding to a region of a naturally-occurring gene or mRNA, but they may still be considered non-sequence specific immunostimulatory nucleic acids.

[0230] In one embodiment, the immunostimulatory nucleic acid or oligonucleotide comprises at least one CpG dinucleotide. The oligonucleotide or CpG dinucleotide may be unmethylated or methylated. In another embodiment, the immunostimulatory nucleic acid comprises at least one CpG dinucleotide having a methylated cytosine. In one embodiment, the nucleic acid comprises a single CpG dinucleotide, wherein the cytosine in the CpG dinucleotide is methylated. In an alternative embodiment, the nucleic acid comprises at least

two CpG dinucleotides, wherein at least one cytosine in the CpG dinucleotides is methylated. In a further embodiment, each cytosine in the CpG dinucleotides present in the sequence is methylated. In another embodiment, the nucleic acid comprises a plurality of CpG dinucleotides, wherein at least one of the CpG dinucleotides comprises a methylated cytosine. Examples of immunostimulatory oligonucleotides suitable for use in the compositions and methods of the present invention are described in PCT Application No. PCT/US08/88676, filed December 31, 2008, PCT Publication Nos. WO 02/069369 and WO 01/15726, U.S. Patent No. 6,406,705, and Raney *et al.*, *J. Pharm. Exper. Ther.*, 298:1185-92 (2001), the disclosures of which are each herein incorporated by reference in their entirety for all purposes. In certain embodiments, the oligonucleotides used in the compositions and methods of the invention have a phosphodiester ("PO") backbone or a phosphorothioate ("PS") backbone, and/or at least one methylated cytosine residue in a CpG motif.

B. Other Active Agents

[0231] In certain embodiments, the active agent associated with the lipid paticles of the invention may comprise one or more therapeutic proteins, polypeptides, or small organic molecules or compounds. Non-limiting examples of such therapeutically effective agents or drugs include oncology drugs (*e.g.*, chemotherapy drugs, hormonal therapaeutic agents, immunotherapeutic agents, radiotherapeutic agents, *etc.*), lipid-lowering agents, anti-viral drugs, anti-inflammatory compounds, antidepressants, stimulants, analgesics, antibiotics, birth control medication, antipyretics, vasodilators, anti-angiogenics, cytovascular agents, signal transduction inhibitors, cardiovascular drugs such as anti-arrhythmic agents, hormones, vasoconstrictors, and steroids. These active agents may be administered alone in the lipid particles of the invention, or in combination (*e.g.*, co-administered) with lipid particles of the invention comprising nucleic acid such as interfering RNA.

[0232] Non-limiting examples of chemotherapy drugs include platinum-based drugs (*e.g.*, oxaliplatin, cisplatin, carboplatin, spiroplatin, iproplatin, satraplatin, *etc.*), alkylating agents (*e.g.*, cyclophosphamide, ifosfamide, chlorambucil, busulfan, melphalan, mechlorethamine, uramustine, thiotepa, nitrosoureas, *etc.*), anti-metabolites (*e.g.*, 5-fluorouracil (5-FU), azathioprine, methotrexate, leucovorin, capecitabine, cytarabine, floxuridine, fludarabine, gemcitabine, pemetrexed, raltitrexed, *etc.*), plant alkaloids (*e.g.*, vincristine, vinblastine, vinorelbine, vindesine, podophyllotoxin, paclitaxel (taxol), docetaxel, *etc.*), topoisomerase inhibitors (*e.g.*, irinotecan (CPT-11; Camptosar), topotecan, amsacrine, etoposide (VP16), etoposide phosphate, teniposide, *etc.*), antitumor antibiotics (*e.g.*, doxorubicin, adriamycin,

daunorubicin, epirubicin, actinomycin, bleomycin, mitomycin, mitoxantrone, plicamycin, *etc.*), tyrosine kinase inhibitors (*e.g.*, gefitinib (Iressa®), sunitinib (Sutent®; SU11248), erlotinib (Tarceva®; OSI-1774), lapatinib (GW572016; GW2016), canertinib (CI 1033), semaxinib (SU5416), vatalanib (PTK787/ZK222584), sorafenib (BAY 43-9006), imatinib (Gleevec®; STI571), dasatinib (BMS-354825), leflunomide (SU101), vandetanib (Zactima™; ZD6474), *etc.*), pharmaceutically acceptable salts thereof, stereoisomers thereof, derivatives thereof, analogs thereof, and combinations thereof.

[0233] Examples of conventional hormonal therapaeutic agents include, without limitation, steroids (*e.g.*, dexamethasone), finasteride, aromatase inhibitors, tamoxifen, and goserelin as well as other gonadotropin-releasing hormone agonists (GnRH).

[0234] Examples of conventional immunotherapeutic agents include, but are not limited to, immunostimulants (*e.g.*, Bacillus Calmette-Guérin (BCG), levamisole, interleukin-2, alpha-interferon, *etc.*), monoclonal antibodies (*e.g.*, anti-CD20, anti-HER2, anti-CD52, anti-HLA-DR, and anti-VEGF monoclonal antibodies), immunotoxins (*e.g.*, anti-CD33 monoclonal antibody-calicheamicin conjugate, anti-CD22 monoclonal antibody-pseudomonas exotoxin conjugate, *etc.*), and radioimmunotherapy (*e.g.*, anti-CD20 monoclonal antibody conjugated to ^{111}In, ^{90}Y, or ^{131}I, *etc.*).

[0235] Examples of conventional radiotherapeutic agents include, but are not limited to, radionuclides such as ^{47}Sc, ^{64}Cu, ^{67}Cu, ^{89}Sr, ^{86}Y, ^{87}Y, ^{90}Y, ^{105}Rh, ^{111}Ag, ^{111}In, ^{117m}Sn, ^{149}Pm, ^{153}Sm, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re, ^{211}At, and ^{212}Bi, optionally conjugated to antibodies directed against tumor antigens.

[0236] Additional oncology drugs that may be used according to the invention include, but are not limited to, alkeran, allopurinol, altretamine, amifostine, anastrozole, araC, arsenic trioxide, bexarotene, biCNU, carmustine, CCNU, celecoxib, cladribine, cyclosporin A, cytosine arabinoside, cytoxan, dexrazoxane, DTIC, estramustine, exemestane, FK506, gemtuzumab-ozogamicin, hydrea, hydroxyurea, idarubicin, interferon, letrozole, leustatin, leuprolide, litretinoin, megastrol, L-PAM, mesna, methoxsalen, mithramycin, nitrogen mustard, pamidronate, Pegademase, pentostatin, porfimer sodium, prednisone, rituxan, streptozocin, STI-571, taxotere, temozolamide, VM-26, toremifene, tretinoin, ATRA, valrubicin, and velban. Other examples of oncology drugs that may be used according to the invention are ellipticin and ellipticin analogs or derivatives, epothilones, intracellular kinase inhibitors, and camptothecins.

[0237] Non-limiting examples of lipid-lowering agents for treating a lipid disease or disorder associated with elevated triglycerides, cholesterol, and/or glucose include statins, fibrates, ezetimibe, thiazolidinediones, niacin, beta-blockers, nitroglycerin, calcium antagonists, fish oil, and mixtures thereof.

[0238] Examples of anti-viral drugs include, but are not limited to, abacavir, aciclovir, acyclovir, adefovir, amantadine, amprenavir, arbidol, atazanavir, atripla, cidofovir, combivir, darunavir, delavirdine, didanosine, docosanol, edoxudine, efavirenz, emtricitabine, enfuvirtide, entecavir, entry inhibitors, famciclovir, fixed dose combinations, fomivirsen, fosamprenavir, foscarnet, fosfonet, fusion inhibitors, ganciclovir, ibacitabine, imunovir, idoxuridine, imiquimod, indinavir, inosine, integrase inhibitors, interferon type III (*e.g.*, IFN-λ molecules such as IFN-λ1, IFN-λ2, and IFN-λ3), interferon type II (*e.g.*, IFN-γ), interferon type I (*e.g.*, IFN-α such as PEGylated IFN-α, IFN-β, IFN-κ, IFN-δ, IFN-ε, IFN-τ, IFN-ω, and IFN-ζ), interferon, lamivudine, lopinavir, loviride, MK-0518, maraviroc, moroxydine, nelfinavir, nevirapine, nexavir, nucleoside analogues, oseltamivir, penciclovir, peramivir, pleconaril, podophyllotoxin, protease inhibitors, reverse transcriptase inhibitors, ribavirin, rimantadine, ritonavir, saquinavir, stavudine, synergistic enhancers, tenofovir, tenofovir disoproxil, tipranavir, trifluridine, trizivir, tromantadine, truvada, valaciclovir, valganciclovir, vicriviroc, vidarabine, viramidine, zalcitabine, zanamivir, zidovudine, pharmaceutically acceptable salts thereof, stereoisomers thereof, derivatives thereof, analogs thereof, and mixtures thereof.

V. Lipid Particles

[0239] The lipid particles of the invention typically comprise an active agent or therapeutic agent, a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles. In some embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.*, by a nuclease or protease. In other embodiments, the lipid particles described herein are substantially non-toxic to mammals such as humans. The lipid particles of the invention typically have a mean diameter of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm.

[0240] In preferred embodiments, the lipid particles of the invention are serum-stable nucleic acid-lipid particles (SNALP) which comprise an interfering RNA (*e.g.*, siRNA,

aiRNA, and/or miRNA), a cationic lipid (*e.g.*, a cationic lipid of Formulas I, II, and/or III), a non-cationic lipid (*e.g.*, cholesterol alone or mixtures of one or more phospholipids and cholesterol), and a conjugated lipid that inhibits aggregation of the particles (*e.g.*, one or more PEG-lipid conjugates). The SNALP may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more unmodified and/or modified interfering RNA molecules. Nucleic acid-lipid particles and their method of preparation are described in, *e.g.*, U.S. Patent Nos. 5,753,613; 5,785,992; 5,705,385; 5,976,567; 5,981,501; 6,110,745; and 6,320,017; and PCT Publication No. WO 96/40964, the disclosures of which are each herein incorporated by reference in their entirety for all purposes.

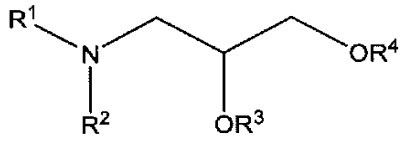
A. Cationic Lipids

[0241] Any of a variety of cationic lipids may be used in the lipid particles of the invention (*e.g.*, SNALP), either alone or in combination with one or more other cationic lipid species or non-cationic lipid species.

[0242] Cationic lipids which are useful in the present invention can be any of a number of lipid species which carry a net positive charge at physiological pH. Such lipids include, but are not limited to, N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3 -(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3.beta.-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',1-2'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-Dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), 1,2-Dilinoleoylcarbamyl-3-dimethylaminopropane (DLinCDAP), and mixtures thereof. A number of these lipids and related analogs have been described in U.S. Patent Publication Nos. 20060083780 and 20060240554; U.S. Patent Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390, the disclosures of which are each herein incorporated by reference in their

entirety for all purposes. Additionally, a number of commercial preparations of cationic lipids are available and can be used in the present invention. These include, *e.g.*, LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, New York, USA); LIPOFECTAMINE® (commercially available cationic liposomes comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic liposomes comprising DOGS from Promega Corp., Madison, Wisconsin, USA).

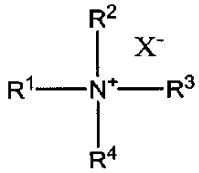
[0243] Additionally, cationic lipids of Formula I having the following structures are useful in the present invention.



(I),

wherein R^1 and R^2 are independently selected and are H or C_1-C_3 alkyls, R^3 and R^4 are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R^3 and R^4 comprises at least two sites of unsaturation. In certain instances, R^3 and R^4 are both the same, *i.e.*, R^3 and R^4 are both linoleyl (C_{18}), *etc.* In certain other instances, R^3 and R^4 are different, *i.e.*, R^3 is tetradectrienyl (C_{14}) and R^4 is linoleyl (C_{18}). In a preferred embodiment, the cationic lipid of Formula I is symmetrical, *i.e.*, R^3 and R^4 are both the same. In another preferred embodiment, both R^3 and R^4 comprise at least two sites of unsaturation. In some embodiments, R^3 and R^4 are independently selected from the group consisting of dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R^3 and R^4 are both linoleyl. In some embodiments, R^3 and R^4comprise at least three sites of unsaturation and are independently selected from, *e.g.*, dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl. In particularly preferred embodiments, the cationic lipid of Formula I is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA) or 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA).

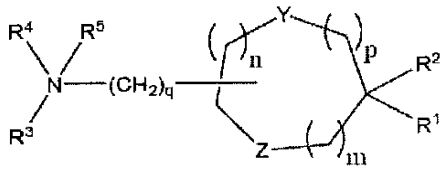
[0244] Furthermore, cationic lipids of Formula II having the following structures are useful in the present invention.



(II),

wherein R^1 and R^2 are independently selected and are H or C_1-C_3 alkyls, R^3 and R^4 are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R^3 and R^4 comprises at least two sites of unsaturation. In certain instances, R^3 and R^4 are both the same, *i.e.*, R^3 and R^4 are both linoleyl (C_{18}), *etc.* In certain other instances, R^3 and R^4 are different, *i.e.*, R^3 is tetradectrienyl (C_{14}) and R^4 is linoleyl (C_{18}). In a preferred embodiment, the cationic lipids of the present invention are symmetrical, *i.e.*, R^3 and R^4 are both the same. In another preferred embodiment, both R^3 and R^4 comprise at least two sites of unsaturation. In some embodiments, R^3 and R^4 are independently selected from the group consisting of dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R^3 and R^4 are both linoleyl. In some embodiments, R^3 and R^4comprise at least three sites of unsaturation and are independently selected from, *e.g.*, dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl.

[0245] Moreover, cationic lipids of Formula III having the following structures (or salts thereof) are useful in the present invention.



(III),

Wherein R^1 and R^2 are either the same or different and independently optionally substituted C_{12}-C_{24} alkyl, optionally substituted C_{12}-C_{24} alkenyl, optionallysubstituted C_{12}-C_{24} alkynyl, or optionally substituted C_{12}-C_{24} acyl; R^3 and R^4 are either the same or different and independently optionally substituted C_1-C_6 alkyl, optionally substituted C_1-C_6 alkenyl, or optionally substituted C_1-C_6 alkynyl or R^3 and R^4 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms chosen from nitrogen and oxygen; R^5 is either absent or hydrogen or C_1-C_6 alkyl to provide a quaternary amine; m, n, and p are either the same or different and independently either 0 or 1 with the proviso that m, n, and p are not simultaneously 0; q is 0, 1, 2, 3, or 4; and Y and Z are either the same or different and independently O, S, or NH.

[0246] In some embodiments, the cationic lipid of Formula III is 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-

[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), or mixtures thereof. In preferred embodiments, the cationic lipid of Formula III is DLin-K-C2-DMA (XTC2).

[0247] The cationic lipid typically comprises from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, or from about 55 mol % to about 65 mol % of the total lipid present in the particle.

[0248] It will be readily apparent to one of skill in the art that depending on the intended use of the particles, the proportions of the components can be varied and the delivery efficiency of a particular formulation can be measured using, *e.g.*, an endosomal release parameter (ERP) assay.

B. Non-Cationic Lipids

[0249] The non-cationic lipids used in the lipid particles of the invention (*e.g.*, SNALP) can be any of a variety of neutral uncharged, zwitterionic, or anionic lipids capable of producing a stable complex.

[0250] Non-limiting examples of non-cationic lipids include phospholipids such as lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol

(POPG), dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), lysophosphatidylcholine, dilinoleoylphosphatidylcholine, and mixtures thereof. Other diacylphosphatidylcholine and diacylphosphatidylethanolamine phospholipids can also be used. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having C_{10}-C_{24} carbon chains, *e.g.*, lauroyl, myristoyl, palmitoyl, stearoyl, or oleoyl.

[0251] Additional examples of non-cationic lipids include sterols such as cholesterol and derivatives thereof such as cholestanol, cholestanone, cholestenone, coprostanol, cholesteryl-2'-hydroxyethyl ether, cholesteryl-4'-hydroxybutyl ether, and mixtures thereof.

[0252] In some embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of cholesterol or a derivative thereof, *e.g.*, a phospholipid-free lipid particle formulation. In other embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of one or more phospholipids, *e.g.*, a cholesterol-free lipid particle formulation. In further embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of a mixture of one or more phospholipids and cholesterol or a derivative thereof.

[0253] Other examples of non-cationic lipids suitable for use in the present invention include nonphosphorous containing lipids such as, *e.g.*, stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerolricinoleate, hexadecyl stereate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyldimethyl ammonium bromide, ceramide, sphingomyelin, and the like.

[0254] In some embodiments, the non-cationic lipid comprises from about 13 mol % to about 49.5 mol %, from about 20 mol % to about 45 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, from about 35 mol % to about 45 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 40 mol %, or from about 30 mol % to about 40 mol % of the total lipid present in the particle.

[0255] In certain embodiments, the cholesterol present in phospholipid-free lipid particles comprises from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 35 mol % to about 45 mol %, or from about 35 mol % to about 40 mol % of the

total lipid present in the particle. As a non-limiting example, a phospholipid-free lipid particle may comprise cholesterol at about 37 mol % of the total lipid present in the particle.

[0256] In certain other embodiments, the cholesterol present in lipid particles containing a mixture of phospholipid and cholesterol comprises from about 30 mol % to about 40 mol %, from about 30 mol % to about 35 mol %, or from about 35 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise cholesterol at about 34 mol % of the total lipid present in the particle.

[0257] In further embodiments, the cholesterol present in lipid particles containing a mixture of phospholipid and cholesterol comprises from about 10 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, or from about 17 mol % to about 23 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise cholesterol at about 20 mol % of the total lipid present in the particle.

[0258] In embodiments where the lipid particles contain a mixture of phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40, 45, 50, 55, or 60 mol % of the total lipid present in the particle. In certain instances, the phospholipid component in the mixture may comprise from about 2 mol % to about 12 mol %, from about 4 mol % to about 10 mol %, from about 5 mol % to about 10 mol %, from about 5 mol % to about 9 mol %, or from about 6 mol % to about 8 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 7 mol % (*e.g.*, in a mixture with about 34 mol % cholesterol) of the total lipid present in the particle. In certain other instances, the phospholipid component in the mixture may comprise from about 10 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, or from about 17 mol % to about 23 mol % of the total lipid present in the particle. As another non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 20 mol % (*e.g.*, in a mixture with about 20 mol % cholesterol) of the total lipid present in the particle.

C. Lipid Conjugate

[0259] In addition to cationic and non-cationic lipids, the lipid particles of the invention (*e.g.*, SNALP) comprise a lipid conjugate. The conjugated lipid is useful in that it prevents the aggregation of particles. Suitable conjugated lipids include, but are not limited to, PEG-

lipid conjugates, ATTA-lipid conjugates, cationic-polymer-lipid conjugates (CPLs), and mixtures thereof. In certain embodiments, the particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate together with a CPL.

[0260] In a preferred embodiment, the lipid conjugate is a PEG-lipid. Examples of PEG-lipids include, but are not limited to, PEG coupled to dialkyloxypropyls (PEG-DAA) as described in, *e.g.*, PCT Publication No. WO 05/026372, PEG coupled to diacylglycerol (PEG-DAG) as described in, *e.g.*, U.S. Patent Publication Nos. 20030077829 and 2005008689, PEG coupled to phospholipids such as phosphatidylethanolamine (PEG-PE), PEG conjugated to ceramides as described in, *e.g.*, U.S. Patent No. 5,885,613, PEG conjugated to cholesterol or a derivative thereof, and mixtures thereof. The disclosures of these patent documents are herein incorporated by reference in their entirety for all purposes. Additional PEG-lipids include, without limitation, PEG-C-DOMG, 2KPEG-DMG, and a mixture thereof.

[0261] PEG is a linear, water-soluble polymer of ethylene PEG repeating units with two terminal hydroxyl groups. PEGs are classified by their molecular weights; for example, PEG 2000 has an average molecular weight of about 2,000 daltons, and PEG 5000 has an average molecular weight of about 5,000 daltons. PEGs are commercially available from Sigma Chemical Co. and other companies and include, for example, the following: monomethoxypolyethylene glycol (MePEG-OH), monomethoxypolyethylene glycol-succinate (MePEG-S), monomethoxypolyethylene glycol-succinimidyl succinate (MePEG-S-NHS), monomethoxypolyethylene glycol-amine (MePEG-NH_2), monomethoxypolyethylene glycol-tresylate (MePEG-TRES), and monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM). Other PEGs such as those described in U.S. Patent Nos. 6,774,180 and 7,053,150 (*e.g.*, mPEG (20 KDa) amine) are also useful for preparing the PEG-lipid conjugates of the present invention. The disclosures of these patents are herein incorporated by reference in their entirety for all purposes. In addition, monomethoxypolyethyleneglycol-acetic acid (MePEG-CH_2COOH) is particularly useful for preparing PEG-lipid conjugates including, *e.g.*, PEG-DAA conjugates.

[0262] The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about

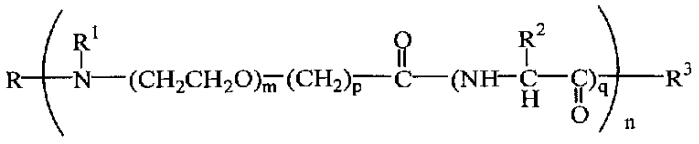
750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

[0263] In certain instances, the PEG can be optionally substituted by an alkyl, alkoxy, acyl, or aryl group. The PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.*, non-ester containing linker moieties and ester-containing linker moieties. In a preferred embodiment, the linker moiety is a non-ester containing linker moiety. As used herein, the term "non-ester containing linker moiety" refers to a linker moiety that does not contain a carboxylic ester bond (-OC(O)-). Suitable non-ester containing linker moieties include, but are not limited to, amido (-C(O)NH-), amino (-NR-), carbonyl (-C(O)-), carbamate (-NHC(O)O-), urea (-NHC(O)NH-), disulphide (-S-S-), ether (-O-), succinyl (-(O)CCH_2CH_2C(O)-), succinamidyl (-NHC(O)CH_2CH_2C(O)NH-), ether, disulphide, as well as combinations thereof (such as a linker containing both a carbamate linker moiety and an amido linker moiety). In a preferred embodiment, a carbamate linker is used to couple the PEG to the lipid.

[0264] In other embodiments, an ester containing linker moiety is used to couple the PEG to the lipid. Suitable ester containing linker moieties include, *e.g.*, carbonate (-OC(O)O-), succinoyl, phosphate esters (-O-(O)POH-O-), sulfonate esters, and combinations thereof.

[0265] Phosphatidylethanolamines having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be conjugated to PEG to form the lipid conjugate. Such phosphatidylethanolamines are commercially available, or can be isolated or synthesized using conventional techniques known to those of skilled in the art. Phosphatidyl-ethanolamines containing saturated or unsaturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20} are preferred. Phosphatidylethanolamines with mono- or diunsaturated fatty acids and mixtures of saturated and unsaturated fatty acids can also be used. Suitable phosphatidylethanolamines include, but are not limited to, dimyristoyl-phosphatidylethanolamine (DMPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE), and distearoyl-phosphatidylethanolamine (DSPE).

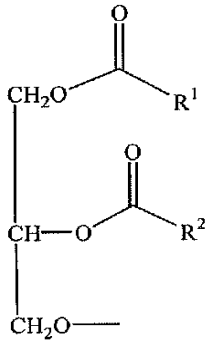
[0266] The term "ATTA" or "polyamide" refers to, without limitation, compounds described in U.S. Patent Nos. 6,320,017 and 6,586,559, the disclosures of which are herein incorporated by reference in their entirety for all purposes. These compounds include a compound having the formula:



(IV),

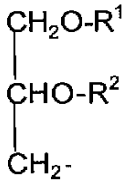
wherein R is a member selected from the group consisting of hydrogen, alkyl and acyl; R^1 is a member selected from the group consisting of hydrogen and alkyl; or optionally, R and R^1 and the nitrogen to which they are bound form an azido moiety; R^2 is a member of the group selected from hydrogen, optionally substituted alkyl, optionally substituted aryl and a side chain of an amino acid; R^3 is a member selected from the group consisting of hydrogen, halogen, hydroxy, alkoxy, mercapto, hydrazino, amino and NR^4R^5, wherein R^4 and R^5 are independently hydrogen or alkyl; n is 4 to 80; m is 2 to 6; p is 1 to 4; and q is 0 or 1. It will be apparent to those of skill in the art that other polyamides can be used in the compounds of the present invention.

[0267] The term "diacylglycerol" refers to a compound having 2 fatty acyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Suitable acyl groups include, but are not limited to, lauryl (C_{12}), myristyl (C_{14}), palmityl (C_{16}), stearyl (C_{18}), and icosyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.*, R^1 and R^2 are both myristyl (*i.e.*, dimyristyl), R^1 and R^2 are both stearyl (*i.e.*, distearyl), *etc.* Diacylglycerols have the following general formula:



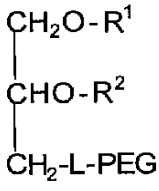
(V).

[0268] The term "dialkyloxypropyl" refers to a compound having 2 alkyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons. The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the following general formula:



(VI).

[0269] In a preferred embodiment, the PEG-lipid is a PEG-DAA conjugate having the following formula:



(VII),

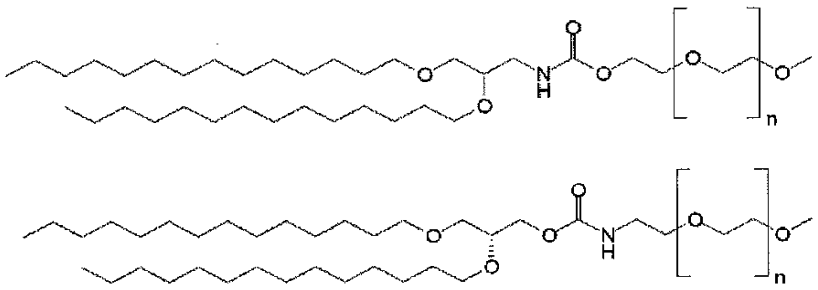
wherein R^1 and R^2 are independently selected and are long-chain alkyl groups having from about 10 to about 22 carbon atoms; PEG is a polyethyleneglycol; and L is a non-ester containing linker moiety or an ester containing linker moiety as described above. The long-chain alkyl groups can be saturated or unsaturated. Suitable alkyl groups include, but are not limited to, lauryl (C_{12}), myristyl (C_{14}), palmityl (C_{16}), stearyl (C_{18}), and icosyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.*, R^1 and R^2 are both myristyl (*i.e.*, dimyristyl), R^1 and R^2 are both stearyl (*i.e.*, distearyl), *etc.*

[0270] In Formula VII above, the PEG has an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG has an average molecular weight of about 2,000 daltons or about 750 daltons. The PEG can be optionally substituted with alkyl, alkoxy, acyl, or aryl. In certain embodiments, the terminal hydroxyl group is substituted with a methoxy or methyl group.

[0271] In a preferred embodiment, "L" is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide linker moiety, a succinamidyl linker moiety, and combinations thereof. In a preferred embodiment, the non-ester containing linker moiety is a carbamate linker moiety (*i.e.*, a PEG-*C*-DAA conjugate). In another preferred embodiment,

the non-ester containing linker moiety is an amido linker moiety (*i.e.*, a PEG-*A*-DAA conjugate). In yet another preferred embodiment, the non-ester containing linker moiety is a succinamidyl linker moiety (*i.e.*, a PEG-*S*-DAA conjugate).

[0272] In particular embodiments, the PEG-lipid conjugate is selected from:



(PEG-C-DMA); and

(PEG-C-DOMG).

[0273] The PEG-DAA conjugates are synthesized using standard techniques and reagents known to those of skill in the art. It will be recognized that the PEG-DAA conjugates will contain various amide, amine, ether, thio, carbamate, and urea linkages. Those of skill in the art will recognize that methods and reagents for forming these bonds are well known and readily available. *See, e.g.*, March, ADVANCED ORGANIC CHEMISTRY (Wiley 1992); Larock, COMPREHENSIVE ORGANIC TRANSFORMATIONS (VCH 1989); and Furniss, VOGEL'S TEXTBOOK OF PRACTICAL ORGANIC CHEMISTRY, 5th ed. (Longman 1989). It will also be appreciated that any functional groups present may require protection and deprotection at different points in the synthesis of the PEG-DAA conjugates. Those of skill in the art will recognize that such techniques are well known. *See, e.g.*, Green and Wuts, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS (Wiley 1991).

[0274] Preferably, the PEG-DAA conjugate is a dilauryloxypropyl (C_{12})-PEG conjugate, dimyristyloxypropyl (C_{14})-PEG conjugate, a dipalmityloxypropyl (C_{16})-PEG conjugate, or a distearyloxypropyl (C_{18})-PEG conjugate. Those of skill in the art will readily appreciate that other dialkyloxypropyls can be used in the PEG-DAA conjugates of the present invention.

[0275] In addition to the foregoing, it will be readily apparent to those of skill in the art that other hydrophilic polymers can be used in place of PEG. Examples of suitable polymers that can be used in place of PEG include, but are not limited to, polyvinylpyrrolidone, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide and polydimethylacrylamide, polylactic acid, polyglycolic acid, and derivatized celluloses such as hydroxymethylcellulose or hydroxyethylcellulose.

[0276] In addition to the foregoing components, the particles (*e.g.*, SNALP or SPLP) of the present invention can further comprise cationic poly(ethylene glycol) (PEG) lipids or CPLs (*see, e.g.*, Chen *et al.*, *Bioconj. Chem.*, 11:433-437 (2000)). Suitable SPLPs and SPLP-CPLs for use in the present invention, and methods of making and using SPLPs and SPLP-CPLs, are disclosed, *e.g.*, in U.S. Patent No. 6,852,334 and PCT Publication No. WO 00/62813, , the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0277] Suitable CPLs include compounds of Formula VIII:

A-W-Y (VIII),

wherein A, W, and Y are as described below.

[0278] With reference to Formula VIII, "A" is a lipid moiety such as an amphipathic lipid, a neutral lipid, or a hydrophobic lipid that acts as a lipid anchor. Suitable lipid examples include, but are not limited to, diacylglycerolyls, dialkylglycerolyls, N-N-dialkylaminos, 1,2-diacyloxy-3-aminopropanes, and 1,2-dialkyl-3-aminopropanes.

[0279] "W" is a polymer or an oligomer such as a hydrophilic polymer or oligomer. Preferably, the hydrophilic polymer is a biocompatable polymer that is nonimmunogenic or possesses low inherent immunogenicity. Alternatively, the hydrophilic polymer can be weakly antigenic if used with appropriate adjuvants. Suitable nonimmunogenic polymers include, but are not limited to, PEG, polyamides, polylactic acid, polyglycolic acid, polylactic acid/polyglycolic acid copolymers, and combinations thereof. In a preferred embodiment, the polymer has a molecular weight of from about 250 to about 7,000 daltons.

[0280] "Y" is a polycationic moiety. The term polycationic moiety refers to a compound, derivative, or functional group having a positive charge, preferably at least 2 positive charges at a selected pH, preferably physiological pH. Suitable polycationic moieties include basic amino acids and their derivatives such as arginine, asparagine, glutamine, lysine, and histidine; spermine; spermidine; cationic dendrimers; polyamines; polyamine sugars; and amino polysaccharides. The polycationic moieties can be linear, such as linear tetralysine, branched or dendrimeric in structure. Polycationic moieties have between about 2 to about 15 positive charges, preferably between about 2 to about 12 positive charges, and more preferably between about 2 to about 8 positive charges at selected pH values. The selection of which polycationic moiety to employ may be determined by the type of particle application which is desired.

[0281] The charges on the polycationic moieties can be either distributed around the entire particle moiety, or alternatively, they can be a discrete concentration of charge density in one particular area of the particle moiety *e.g.*, a charge spike. If the charge density is distributed

on the particle, the charge density can be equally distributed or unequally distributed. All variations of charge distribution of the polycationic moiety are encompassed by the present invention.

[0282] The lipid "A" and the nonimmunogenic polymer "W" can be attached by various methods and preferably by covalent attachment. Methods known to those of skill in the art can be used for the covalent attachment of "A" and "W." Suitable linkages include, but are not limited to, amide, amine, carboxyl, carbonate, carbamate, ester, and hydrazone linkages. It will be apparent to those skilled in the art that "A" and "W" must have complementary functional groups to effectuate the linkage. The reaction of these two groups, one on the lipid and the other on the polymer, will provide the desired linkage. For example, when the lipid is a diacylglycerol and the terminal hydroxyl is activated, for instance with NHS and DCC, to form an active ester, and is then reacted with a polymer which contains an amino group, such as with a polyamide (*see, e.g.,* U.S. Patent Nos. 6,320,017 and 6,586,559, the disclosures of which are herein incorporated by reference in their entirety for all purposes), an amide bond will form between the two groups.

[0283] In certain instances, the polycationic moiety can have a ligand attached, such as a targeting ligand or a chelating moiety for complexing calcium. Preferably, after the ligand is attached, the cationic moiety maintains a positive charge. In certain instances, the ligand that is attached has a positive charge. Suitable ligands include, but are not limited to, a compound or device with a reactive functional group and include lipids, amphipathic lipids, carrier compounds, bioaffinity compounds, biomaterials, biopolymers, biomedical devices, analytically detectable compounds, therapeutically active compounds, enzymes, peptides, proteins, antibodies, immune stimulators, radiolabels, fluorogens, biotin, drugs, haptens, DNA, RNA, polysaccharides, liposomes, virosomes, micelles, immunoglobulins, functional groups, other targeting moieties, or toxins.

[0284] The lipid conjugate (*e.g.,* PEG-lipid) typically comprises from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 0.6 mol % to about 1.9 mol %, from about 0.7 mol % to about 1.8 mol %, from about 0.8 mol % to about 1.7 mol %, from about 0.9 mol % to about 1.6 mol %, from about 0.9 mol % to about I.8 mol %, from about 1 mol % to about 1.8 mol %, from about 1 mol % to about 1.7 mol %, from about 1.2 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.7 mol %, from about 1.3 mol % to about 1.6 mol %, or from about 1.4 mol % to about 1.5 mol % of the total lipid present in the particle.

[0285] One of ordinary skill in the art will appreciate that the concentration of the lipid conjugate can be varied depending on the lipid conjugate employed and the rate at which the nucleic acid-lipid particle is to become fusogenic.

[0286] By controlling the composition and concentration of the lipid conjugate, one can control the rate at which the lipid conjugate exchanges out of the nucleic acid-lipid particle and, in turn, the rate at which the nucleic acid-lipid particle becomes fusogenic. For instance, when a PEG-phosphatidylethanolamine conjugate or a PEG-ceramide conjugate is used as the lipid conjugate, the rate at which the nucleic acid-lipid particle becomes fusogenic can be varied, for example, by varying the concentration of the lipid conjugate, by varying the molecular weight of the PEG, or by varying the chain length and degree of saturation of the acyl chain groups on the phosphatidylethanolamine or the ceramide. In addition, other variables including, for example, pH, temperature, ionic strength, *etc.* can be used to vary and/or control the rate at which the nucleic acid-lipid particle becomes fusogenic. Other methods which can be used to control the rate at which the nucleic acid-lipid particle becomes fusogenic will become apparent to those of skill in the art upon reading this disclosure.

VI. Preparation of Lipid Particles

[0287] The lipid particles of the present invention, *e.g.,* SNALP, in which an active agent or therapeutic agent such as an interfering RNA is encapsulated in a lipid bilayer and is protected from degradation, can be formed by any method known in the art including, but not limited to, a continuous mixing method or a direct dilution process.

[0288] In preferred embodiments, the cationic lipids are lipids of Formula I, II, and III, or combinations thereof. In other preferred embodiments, the non-cationic lipids are egg sphingomyelin (ESM), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), dipalmitoyl-phosphatidylcholine (DPPC), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, 14:0 PE (1,2-dimyristoyl-phosphatidylethanolamine (DMPE)), 16:0 PE (1,2-dipalmitoyl-phosphatidylethanolamine (DPPE)), 18:0 PE (1,2-distearoyl-phosphatidylethanolamine (DSPE)), 18:1 PE (1,2-dioleoyl-phosphatidylethanolamine (DOPE)), 18:1 trans PE (1,2-dielaidoyl-phosphatidylethanolamine (DEPE)), 18:0-18:1 PE (1-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE)), 16:0-18:1 PE (1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)), polyethylene glycol-based polymers (*e.g.,* PEG 2000,

PEG 5000, PEG-modified diacylglycerols, or PEG-modified dialkyloxypropyls), cholesterol, or combinations thereof.

[0289] In certain embodiments, the present invention provides for SNALP produced via a continuous mixing method, *e.g.*, a process that includes providing an aqueous solution comprising a nucleic acid such as an interfering RNA in a first reservoir, providing an organic lipid solution in a second reservoir, and mixing the aqueous solution with the organic lipid solution such that the organic lipid solution mixes with the aqueous solution so as to substantially instantaneously produce a liposome encapsulating the nucleic acid (*e.g.*, interfering RNA). This process and the apparatus for carrying this process are described in detail in U.S. Patent Publication No. 20040142025, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0290] The action of continuously introducing lipid and buffer solutions into a mixing environment, such as in a mixing chamber, causes a continuous dilution of the lipid solution with the buffer solution, thereby producing a liposome substantially instantaneously upon mixing. As used herein, the phrase "continuously diluting a lipid solution with a buffer solution" (and variations) generally means that the lipid solution is diluted sufficiently rapidly in a hydration process with sufficient force to effectuate vesicle generation. By mixing the aqueous solution comprising a nucleic acid with the organic lipid solution, the organic lipid solution undergoes a continuous stepwise dilution in the presence of the buffer solution (*i.e.*, aqueous solution) to produce a nucleic acid-lipid particle.

[0291] The SNALP formed using the continuous mixing method typically have a size of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 nm to about 90 nm. The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0292] In another embodiment, the present invention provides for SNALP produced via a direct dilution process that includes forming a liposome solution and immediately and directly introducing the liposome solution into a collection vessel containing a controlled amount of dilution buffer. In preferred aspects, the collection vessel includes one or more elements configured to stir the contents of the collection vessel to facilitate dilution. In one aspect, the amount of dilution buffer present in the collection vessel is substantially equal to the volume of liposome solution introduced thereto. As a non-limiting example, a liposome solution in 45% ethanol when introduced into the collection vessel containing an equal volume of dilution buffer will advantageously yield smaller particles.

[0293] In yet another embodiment, the present invention provides for SNALP produced via a direct dilution process in which a third reservoir containing dilution buffer is fluidly coupled to a second mixing region. In this embodiment, the liposome solution formed in a first mixing region is immediately and directly mixed with dilution buffer in the second mixing region. In preferred aspects, the second mixing region includes a T-connector arranged so that the liposome solution and the dilution buffer flows meet as opposing 180° flows; however, connectors providing shallower angles can be used, *e.g.*, from about 27° to about 180°. A pump mechanism delivers a controllable flow of buffer to the second mixing region. In one aspect, the flow rate of dilution buffer provided to the second mixing region is controlled to be substantially equal to the flow rate of liposome solution introduced thereto from the first mixing region. This embodiment advantageously allows for more control of the flow of dilution buffer mixing with the liposome solution in the second mixing region, and therefore also the concentration of liposome solution in buffer throughout the second mixing process. Such control of the dilution buffer flow rate advantageously allows for small particle size formation at reduced concentrations.

[0294] These processes and the apparatuses for carrying out these direct dilution processes are described in detail in U.S. Patent Publication No. 20070042031, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0295] The SNALP formed using the direct dilution process typically have a size of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 nm to about 90 nm. The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0296] If needed, the lipid particles of the invention (*e.g.*, SNALP) can be sized by any of the methods available for sizing liposomes. The sizing may be conducted in order to achieve a desired size range and relatively narrow distribution of particle sizes.

[0297] Several techniques are available for sizing the particles to a desired size. One sizing method, used for liposomes and equally applicable to the present particles, is described in U.S. Patent No. 4,737,323, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Sonicating a particle suspension either by bath or probe sonication produces a progressive size reduction down to particles of less than about 50 nm in size. Homogenization is another method which relies on shearing energy to fragment larger particles into smaller ones. In a typical homogenization procedure, particles are recirculated through a standard emulsion homogenizer until selected particle sizes, typically between

about 60 and about 80 nm, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination, or QELS.

[0298] Extrusion of the particles through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing particle sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired particle size distribution is achieved. The particles may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in size.

[0299] In some embodiments, the nucleic acids in the SNALP are precondensed as described in, *e.g.*, U.S. Patent Application No. 09/744,103, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0300] In other embodiments, the methods will further comprise adding non-lipid polycations which are useful to effect the lipofection of cells using the present compositions. Examples of suitable non-lipid polycations include, hexadimethrine bromide (sold under the brandname POLYBRENE®, from Aldrich Chemical Co., Milwaukee, Wisconsin, USA) or other salts of hexadimethrine. Other suitable polycations include, for example, salts of poly-L-ornithine, poly-L-arginine, poly-L-lysine, poly-D-lysine, polyallylamine, and polyethyleneimine. Addition of these salts is preferably after the particles have been formed.

[0301] In some embodiments, the nucleic acid to lipid ratios (mass/mass ratios) in a formed SNALP will range from about 0.01 to about 0.2, from about 0.02 to about 0.1, from about 0.03 to about 0.1, or from about 0.01 to about 0.08. The ratio of the starting materials also falls within this range. In other embodiments, the SNALP preparation uses about 400 μg nucleic acid per 10 mg total lipid or a nucleic acid to lipid mass ratio of about 0.01 to about 0.08 and, more preferably, about 0.04, which corresponds to 1.25 mg of total lipid per 50 μg of nucleic acid. In other preferred embodiments, the particle has a nucleic acid:lipid mass ratio of about 0.08.

[0302] In other embodiments, the lipid to nucleic acid ratios (mass/mass ratios) in a formed SNALP will range from about 1 (1:1) to about 100 (100:1), from about 5 (5:1) to about 100 (100:1), from about 1 (1:1) to about 50 (50:1), from about 2 (2:1) to about 50 (50:1), from about 3 (3:1) to about 50 (50:1), from about 4 (4:1) to about 50 (50:1), from about 5 (5:1) to about 50 (50:1), from about 1 (1:1) to about 25 (25:1), from about 2 (2:1) to about 25 (25:1), from about 3 (3:1) to about 25 (25:1), from about 4 (4:1) to about 25 (25:1), from about 5 (5:1) to about 25 (25:1), from about 5 (5:1) to about 20 (20:1), from about 5 (5:1) to about 15 (15:1), from about 5 (5:1) to about 10 (10:1), about 5 (5:1), 6 (6:1), 7 (7:1), 8 (8:1), 9 (9:1),

10 (10:1), 11 (11:1), 12 (12:1), 13 (13:1), 14 (14:1), or 15 (15:1). The ratio of the starting materials also falls within this range.

[0303] As previously discussed, the conjugated lipid may further include a CPL. A variety of general methods for making SNALP-CPLs (CPL-containing SNALP) are discussed herein. Two general techniques include "post-insertion" technique, that is, insertion of a CPL into, for example, a pre-formed SNALP, and the "standard" technique, wherein the CPL is included in the lipid mixture during, for example, the SNALP formation steps. The post-insertion technique results in SNALP having CPLs mainly in the external face of the SNALP bilayer membrane, whereas standard techniques provide SNALP having CPLs on both internal and external faces. The method is especially useful for vesicles made from phospholipids (which can contain cholesterol) and also for vesicles containing PEG-lipids (such as PEG-DAAs and PEG-DAGs). Methods of making SNALP-CPL, are taught, for example, in U.S. Patent Nos. 5,705,385; 6,586,410; 5,981,501; 6,534,484; and 6,852,334; U.S. Patent Publication No. 20020072121; and PCT Publication No. WO 00/62813, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

VII. Kits

[0304] The present invention also provides lipid particles (*e.g.*, SNALP) in kit form. The kit may comprise a container which is compartmentalized for holding the various elements of the lipid particles (*e.g.*, the active agents or therapeutic agents such as nucleic acids and the individual lipid components of the particles). In some embodiments, the kit may further comprise an endosomal membrane destabilizer (*e.g.*, calcium ions). The kit typically contains the lipid particle compositions of the present invention, preferably in dehydrated form, with instructions for their rehydration and administration.

[0305] As explained herein, the lipid particles of the invention (*e.g.*, SNALP) can be tailored to preferentially target particular tissues, organs, or tumors of interest. In certain instances, preferential targeting of lipid particles such as SNALP may be carried out by controlling the composition of the particle itself. For instance, as set forth in Example 11, it has been found that the 1:57 PEG-cDSA SNALP formulation can be used to preferentially target tumors outside of the liver, whereas the 1:57 PEG-cDMA SNALP formulation can be used to preferentially target the liver (including liver tumors).

[0306] In certain other instances, it may be desirable to have a targeting moiety attached to the surface of the lipid particle to further enhance the targeting of the particle. Methods of

attaching targeting moieties (*e.g.*, antibodies, proteins, *etc.*) to lipids (such as those used in the present particles) are known to those of skill in the art.

VII. Administration of Lipid Particles

[0307] Once formed, the lipid particles of the invention (*e.g.*, SNALP) are useful for the introduction of active agents or therapeutic agents (*e.g.*, nucleic acids such as interfering RNA) into cells. Accordingly, the present invention also provides methods for introducing an active agent or therapeutic agent such as a nucleic acid (*e.g.*, interfering RNA) into a cell. The methods are carried out *in vitro* or *in vivo* by first forming the particles as described above and then contacting the particles with the cells for a period of time sufficient for delivery of the active agent or therapeutic agent to the cells to occur.

[0308] The lipid particles of the invention (*e.g.*, SNALP) can be adsorbed to almost any cell type with which they are mixed or contacted. Once adsorbed, the particles can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the active agent or therapeutic agent (*e.g.*, nucleic acid) portion of the particle can take place via any one of these pathways. In particular, when fusion takes place, the particle membrane is integrated into the cell membrane and the contents of the particle combine with the intracellular fluid.

[0309] The lipid particles of the invention (*e.g.*, SNALP) can be administered either alone or in a mixture with a pharmaceutically-acceptable carrier (*e.g.*, physiological saline or phosphate buffer) selected in accordance with the route of administration and standard pharmaceutical practice. Generally, normal buffered saline (*e.g.*, 135-150 mM NaCl) will be employed as the pharmaceutically-acceptable carrier. Other suitable carriers include, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Additional suitable carriers are described in, *e.g.*, REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

[0310] The pharmaceutically-acceptable carrier is generally added following particle formation. Thus, after the particle is formed, the particle can be diluted into pharmaceutically-acceptable carriers such as normal buffered saline.

[0311] The concentration of particles in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.05%, usually at or at least about 2 to 5%, to as much as about 10 to 90% by weight, and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, particles composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration.

[0312] The pharmaceutical compositions of the present invention may be sterilized by conventional, well-known sterilization techniques. Aqueous solutions can be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions can contain pharmaceutically-acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, and calcium chloride. Additionally, the particle suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alphatocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

A. *In vivo* Administration

[0313] Systemic delivery for *in vivo* therapy, *e.g.*, delivery of a therapeutic nucleic acid to a distal target cell via body systems such as the circulation, has been achieved using nucleic acid-lipid particles such as those described in PCT Publication Nos. WO 05/007196, WO 05/121348, WO 05/120152, and WO 04/002453, the disclosures of which are herein incorporated by reference in their entirety for all purposes. The present invention also provides fully encapsulated lipid particles that protect the nucleic acid from nuclease degradation in serum, are nonimmunogenic, are small in size, and are suitable for repeat dosing.

[0314] For *in vivo* administration, administration can be in any manner known in the art, *e.g.*, by injection, oral administration, inhalation (*e.g.*, intransal or intratracheal), transdermal

application, or rectal administration. Administration can be accomplished via single or divided doses. The pharmaceutical compositions can be administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. In some embodiments, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection (*see, e.g.*, U.S. Patent No. 5,286,634). Intracellular nucleic acid delivery has also been discussed in Straubringer *et al.*, *Methods Enzymol.*, 101:512 (1983); Mannino *et al.*, *Biotechniques,* 6:682 (1988); Nicolau *et al.*, *Crit. Rev. Ther. Drug Carrier Syst.*, 6:239 (1989); and Behr, *Acc. Chem. Res.*, 26:274 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, U.S. Patent Nos. 3,993,754; 4,145,410; 4,235,871; 4,224,179; 4,522,803; and 4,588,578. The lipid particles can be administered by direct injection at the site of disease or by injection at a site distal from the site of disease (*see, e.g.*, Culver, HUMAN GENE THERAPY, MaryAnn Liebert, Inc., Publishers, New York. pp.70-71(1994)). The disclosures of the above-described references are herein incorporated by reference in their entirety for all purposes.

[0315] The compositions of the present invention, either alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, they can be "nebulized") to be administered via inhalation (*e.g.*, intranasally or intratracheally) (*see*, Brigham *et al.*, *Am. J. Sci.*, 298:278 (1989)). Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

[0316] In certain embodiments, the pharmaceutical compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering nucleic acid compositions directly to the lungs via nasal aerosol sprays have been described, *e.g.*, in U.S. Patent Nos. 5,756,353 and 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins and lysophosphatidyl-glycerol compounds (U.S. Patent 5,725,871) are also well-known in the pharmaceutical arts. Similarly, transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U.S. Patent No. 5,780,045. The disclosures of the above-described patents are herein incorporated by reference in their entirety for all purposes.

[0317] Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers,

and preservatives. In the practice of this invention, compositions are preferably administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically, or intrathecally.

[0318] Generally, when administered intravenously, the lipid particle formulations are formulated with a suitable pharmaceutical carrier. Many pharmaceutically acceptable carriers may be employed in the compositions and methods of the present invention. Suitable formulations for use in the present invention are found, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). A variety of aqueous carriers may be used, for example, water, buffered water, 0.4% saline, 0.3% glycine, and the like, and may include glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Generally, normal buffered saline (135-150 mM NaCl) will be employed as the pharmaceutically acceptable carrier, but other suitable carriers will suffice. These compositions can be sterilized by conventional liposomal sterilization techniques, such as filtration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, *etc.* These compositions can be sterilized using the techniques referred to above or, alternatively, they can be produced under sterile conditions. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

[0319] In certain applications, the lipid particles disclosed herein may be delivered via oral administration to the individual. The particles may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, pills, lozenges, elixirs, mouthwash, suspensions, oral sprays, syrups, wafers, and the like (*see, e.g.,* U.S. Patent Nos. 5,641,515, 5,580,579, and 5,792,451, the disclosures of which are herein incorporated by reference in their entirety for all purposes). These oral dosage forms may also contain the following: binders, gelatin; excipients, lubricants, and/or flavoring agents. When the unit dosage form is a capsule, it may contain, in addition to the materials described above, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. Of course, any material used in preparing any unit dosage form should be pharmaceutically pure and substantially non-toxic in the amounts employed.

[0320] Typically, these oral formulations may contain at least about 0.1% of the lipid particles or more, although the percentage of the particles may, of course, be varied and may conveniently be between about 1% or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of particles in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[0321] Formulations suitable for oral administration can consist of: (a) liquid solutions, such as an effective amount of a packaged therapeutic agent such as nucleic acid (*e.g.*, interfering RNA) suspended in diluents such as water, saline, or PEG 400; (b) capsules, sachets, or tablets, each containing a predetermined amount of a therapeutic agent such as nucleic acid (*e.g.*, interfering RNA), as liquids, solids, granules, or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise a therapeutic agent such as nucleic acid (*e.g.*, interfering RNA) in a flavor, *e.g.*, sucrose, as well as pastilles comprising the therapeutic agent in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the therapeutic agent, carriers known in the art.

[0322] In another example of their use, lipid particles can be incorporated into a broad range of topical dosage forms. For instance, a suspension containing nucleic acid-lipid particles such as SNALP can be formulated and administered as gels, oils, emulsions, topical creams, pastes, ointments, lotions, foams, mousses, and the like.

[0323] When preparing pharmaceutical preparations of the lipid particles of the invention, it is preferable to use quantities of the particles which have been purified to reduce or eliminate empty particles or particles with therapeutic agents such as nucleic acid associated with the external surface.

[0324] The methods of the present invention may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as primates (*e.g.*, humans and chimpanzees as well as other nonhuman primates), canines, felines, equines, bovines, ovines, caprines, rodents (*e.g.*, rats and mice), lagomorphs, and swine.

[0325] The amount of particles administered will depend upon the ratio of therapeutic agent (*e.g.*, nucleic acid) to lipid, the particular therapeutic agent (*e.g.*, nucleic acid) used, the disease or disorder being treated, the age, weight, and condition of the patient, and the judgment of the clinician, but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight, or about 10^8-10^{10} particles per administration (*e.g.*, injection).

B. *In vitro* Administration

[0326] For *in vitro* applications, the delivery of therapeutic agents such as nucleic acids (*e.g.*, interfering RNA) can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells are animal cells, more preferably mammalian cells, and most preferably human cells.

[0327] Contact between the cells and the lipid particles, when carried out *in vitro*, takes place in a biologically compatible medium. The concentration of particles varies widely depending on the particular application, but is generally between about 1 μmol and about 10 mmol. Treatment of the cells with the lipid particles is generally carried out at physiological temperatures (about 37°C) for periods of time of from about 1 to 48 hours, preferably of from about 2 to 4 hours.

[0328] In one group of preferred embodiments, a lipid particle suspension is added to 60-80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/ml, more preferably about 2 x 10^4 cells/ml. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2 μg/ml, more preferably about 0.1 μg/ml.

[0329] Using an Endosomal Release Parameter (ERP) assay, the delivery efficiency of the SNALP or other lipid particle of the invention can be optimized. An ERP assay is described in detail in U.S. Patent Publication No. 20030077829, the disclosure of which is herein incorporated by reference in its entirety for all purposes. More particularly, the purpose of an ERP assay is to distinguish the effect of various cationic lipids and helper lipid components of SNALP based on their relative effect on binding/uptake or fusion with/destabilization of the endosomal membrane. This assay allows one to determine quantitatively how each component of the SNALP or other lipid particle affects delivery efficiency, thereby

optimizing the SNALP or other lipid particle. Usually, an ERP assay measures expression of a reporter protein (*e.g.*, luciferase, β-galactosidase, green fluorescent protein (GFP), *etc.*), and in some instances, a SNALP formulation optimized for an expression plasmid will also be appropriate for encapsulating an interfering RNA. In other instances, an ERP assay can be adapted to measure downregulation of transcription or translation of a target sequence in the presence or absence of an interfering RNA (*e.g.*, siRNA). By comparing the ERPs for each of the various SNALP or other lipid particles, one can readily determine the optimized system, *e.g.*, the SNALP or other lipid particle that has the greatest uptake in the cell.

C. Cells for Delivery of Lipid Particles

[0330] The compositions and methods of the present invention are used to treat a wide variety of cell types, *in vivo* and *in vitro*. Suitable cells include, *e.g.*, hematopoietic precursor (stem) cells, fibroblasts, keratinocytes, hepatocytes, endothelial cells, skeletal and smooth muscle cells, osteoblasts, neurons, quiescent lymphocytes, terminally differentiated cells, slow or noncycling primary cells, parenchymal cells, lymphoid cells, epithelial cells, bone cells, and the like. In preferred embodiments, an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) is delivered to cancer cells such as, *e.g.*, lung cancer cells, colon cancer cells, rectal cancer cells, anal cancer cells, bile duct cancer cells, small intestine cancer cells, stomach (gastric) cancer cells, esophageal cancer cells, gallbladder cancer cells, liver cancer cells, pancreatic cancer cells, appendix cancer cells, breast cancer cells, ovarian cancer cells, cervical cancer cells, prostate cancer cells, renal cancer cells, cancer cells of the central nervous system, glioblastoma tumor cells, skin cancer cells, lymphoma cells, choriocarcinoma tumor cells, head and neck cancer cells, osteogenic sarcoma tumor cells, and blood cancer cells.

[0331] *In vivo* delivery of lipid particles such as SNALP encapsulating an interfering RNA (*e.g.*, siRNA) is suited for targeting cells of any cell type. The methods and compositions can be employed with cells of a wide variety of vertebrates, including mammals, such as, *e.g*, canines, felines, equines, bovines, ovines, caprines, rodents (*e.g.*, mice, rats, and guinea pigs), lagomorphs, swine, and primates (*e.g.* monkeys, chimpanzees, and humans).

[0332] To the extent that tissue culture of cells may be required, it is well-known in the art. For example, Freshney, Culture of Animal Cells, a Manual of Basic Technique, 3rd Ed., Wiley-Liss, New York (1994), Kuchler *et al.*, Biochemical Methods in Cell Culture and Virology, Dowden, Hutchinson and Ross, Inc. (1977), and the references cited therein

provide a general guide to the culture of cells. Cultured cell systems often will be in the form of monolayers of cells, although cell suspensions are also used.

D. Detection of Lipid Particles

[0333] In some embodiments, the lipid particles of the present invention (*e.g.*, SNALP) are detectable in the subject at about 1, 2, 3, 4, 5, 6, 7, 8 or more hours. In other embodiments, the lipid particles of the present invention (*e.g.*, SNALP) are detectable in the subject at about 8, 12, 24, 48, 60, 72, or 96 hours, or about 6, 8, 10, 12, 14, 16, 18, 19, 22, 24, 25, or 28 days after administration of the particles. The presence of the particles can be detected in the cells, tissues, or other biological samples from the subject. The particles may be detected, *e.g.*, by direct detection of the particles, detection of a therapeutic nucleic acid such as an interfering RNA (*e.g.*, siRNA) sequence, detection of the target sequence of interest (*i.e.*, by detecting expression or reduced expression of the sequence of interest), or a combination thereof.

1. Detection of Particles

[0334] Lipid particles of the invention such as SNALP can be detected using any method known in the art. For example, a label can be coupled directly or indirectly to a component of the lipid particle using methods well-known in the art. A wide variety of labels can be used, with the choice of label depending on sensitivity required, ease of conjugation with the lipid particle component, stability requirements, and available instrumentation and disposal provisions. Suitable labels include, but are not limited to, spectral labels such as fluorescent dyes (*e.g.*, fluorescein and derivatives, such as fluorescein isothiocyanate (FITC) and Oregon Green™; rhodamine and derivatives such Texas red, tetrarhodimine isothiocynate (TRITC), *etc.*, digoxigenin, biotin, phycoerythrin, AMCA, CyDyes™, and the like; radiolabels such as ^{3}H, ^{125}I, ^{35}S, ^{14}C, ^{32}P, ^{33}P, *etc.*; enzymes such as horse radish peroxidase, alkaline phosphatase, *etc.*; spectral colorimetric labels such as colloidal gold or colored glass or plastic beads such as polystyrene, polypropylene, latex, *etc.* The label can be detected using any means known in the art.

2. Detection of Nucleic Acids

[0335] Nucleic acids (*e.g.*, interfering RNA) are detected and quantified herein by any of a number of means well-known to those of skill in the art. The detection of nucleic acids may proceed by well-known methods such as Southern analysis, Northern analysis, gel electrophoresis, PCR, radiolabeling, scintillation counting, and affinity chromatography. Additional analytic biochemical methods such as spectrophotometry, radiography,

electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography may also be employed.

[0336] The selection of a nucleic acid hybridization format is not critical. A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays. Hybridization techniques are generally described in, *e.g.*, "Nucleic Acid Hybridization, A Practical Approach," Eds. Hames and Higgins, IRL Press (1985).

[0337] The sensitivity of the hybridization assays may be enhanced through use of a nucleic acid amplification system which multiplies the target nucleic acid being detected. *In vitro* amplification techniques suitable for amplifying sequences for use as molecular probes or for generating nucleic acid fragments for subsequent subcloning are known. Examples of techniques sufficient to direct persons of skill through such *in vitro* amplification methods, including the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Qβ-replicase amplification and other RNA polymerase mediated techniques (*e.g.*, NASBA™) are found in Sambrook *et al.*, In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (2000); and Ausubel *et al.*, SHORT PROTOCOLS IN MOLECULAR BIOLOGY, eds., Current Protocols, Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (2002); as well as U.S. Patent No. 4,683,202; PCR Protocols, A Guide to Methods and Applications (Innis *et al.* eds.) Academic Press Inc. San Diego, CA (1990); Arnheim & Levinson (October 1, 1990), *C&EN* 36; The *Journal Of NIH Research*, 3:81 (1991); Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:1173 (1989); Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:1874 (1990); Lomell *et al.*, *J. Clin. Chem.*, 35:1826 (1989); Landegren *et al.*, *Science*, 241:1077 (1988); Van Brunt, *Biotechnology*, 8:291 (1990); Wu and Wallace, *Gene*, 4:560 (1989); Barringer *et al.*, *Gene*, 89:117 (1990); and Sooknanan and Malek, *Biotechnology*, 13:563 (1995). Improved methods of cloning *in vitro* amplified nucleic acids are described in U.S. Pat. No. 5,426,039. Other methods described in the art are the nucleic acid sequence based amplification (NASBA™, Cangene, Mississauga, Ontario) and Qβ-replicase systems. These systems can be used to directly identify mutants where the PCR or LCR primers are designed to be extended or ligated only when a select sequence is present. Alternatively, the select sequences can be generally amplified using, for example, nonspecific PCR primers and the amplified target region later probed for a specific sequence indicative of a mutation. The disclosures of the above-described references are herein incorporated by reference in their entirety for all purposes.

[0338] Nucleic acids for use as probes, *e.g.*, in *in vitro* amplification methods, for use as gene probes, or as inhibitor components are typically synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage *et al.*, *Tetrahedron Letts.*, 22:1859 1862 (1981), *e.g.*, using an automated synthesizer, as described in Needham VanDevanter *et al.*, *Nucleic Acids Res.*, 12:6159 (1984). Purification of ploynucleotides, where necessary, is typically performed by either native acrylamide gel electrophoresis or by anion exchange HPLC as described in Pearson *et al.*, *J. Chrom.*, 255:137 149 (1983). The sequence of the synthetic poluyucleotides can be verified using the chemical degradation method of Maxam and Gilbert (1980) in Grossman and Moldave (eds.) Academic Press, New York, *Methods in Enzymology*, 65:499.

[0339] An alternative means for determining the level of transcription is *in situ* hybridization. *In situ* hybridization assays are well-known and are generally described in Angerer *et al.*, *Methods Enzymol.*, 152:649 (1987). In an *in situ* hybridization assay, cells are fixed to a solid support, typically a glass slide. If DNA is to be probed, the cells are denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of specific probes that are labeled. The probes are preferably labeled with radioisotopes or fluorescent reporters.

VIII. Examples

[0340] The present invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

Example 1. Materials and Methods.

[0341] ***siRNA***: All siRNA molecules used in these studies were chemically synthesized by the University of Calgary (Calgary, AB) or Dharmacon Inc. (Lafayette, CO). The siRNAs were desalted and annealed using standard procedures.

[0342] ***Lipid Encapsulation of siRNA***: In some embodiments, siRNA molecules were encapsulated into nucleic acid-lipid particles composed of the following lipids: the lipid conjugate PEG-cDMA (3-N-[(-Methoxypoly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxypropylamine); the cationic lipid DLinDMA (1,2-Dilinoleyloxy-3-(N,N-dimethyl)aminopropane); the phospholipid DPPC (1,2-Dipalmitoyl-sn-glycero-3-

phosphocholine; Avanti Polar Lipids; Alabaster, AL); and synthetic cholesterol (Sigma-Aldrich Corp.; St. Louis, MO) in the molar ratio 1.4:57.1:7.1:34.3, respectively. In other words, siRNAs were encapsulated into SNALP of the following "1:57" formulation: 1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol. In other embodiments, siRNA molecules were encapsulated into phospholipid-free SNALP composed of the following lipids: the lipid conjugate PEG-cDMA; the cationic lipid DLinDMA; and synthetic cholesterol in the molar ratio 1.5:61.5:36.9, respectively. In other words, siRNAs were encapsulated into phospholipid-free SNALP of the following "1:62" formulation: 1.5% PEG-cDMA; 61.5% DLinDMA; and 36.9% cholesterol. For vehicle controls, empty particles with identical lipid composition were formed in the absence of siRNA. It should be understood that the 1:57 formulation and 1:62 formulation are target formulations, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the formulation may vary. Typically, in the 1:57 formulation, the amount of cationic lipid will be 57 mol % ± 5 mol %, and the amount of lipid conjugate will be 1.5 mol % ± 0.5 mol %, with the balance of the 1:57 formulation being made up of non-cationic lipid (*e.g.*, phospholipid, cholesterol, or a mixture of the two). Similarly, in the 1:62 formulation, the amount of cationic lipid will be 62 mol % ± 5 mol %, and the amount of lipid conjugate will be 1.5 mol % ± 0.5 mol %, with the balance of the 1:62 formulation being made up of the non-cationic lipid (*e.g.*, cholesterol).

Example 2. Eg5 siRNA Formulated as 1:57 SNALP Are Potent Inhibitors of Cell Growth *in vitro*.

[0343] SNALP formulations were prepared with an siRNA targeting Eg5 as the nucleic acid component. Eg5 is a member of kinesin-related proteins that are involved in functions related to movements of organelles, microtubules, or chromosomes along microtubules. These functions include axonal transport, microtubule sliding during nuclear fusion or division, and chromosome disjunction during meiosis and early mitosis. Eg5 plays a critical role in mitosis of mammalian cells. The Eg5 siRNA used in this study is provided in Table 1. The modifications involved introducing 2'OMe-uridine at selected positions in the sense and antisense strands of the Eg5 2263 siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 1. siRNA duplex comprising sense and antisense Eg5 RNA polynucleotides.

Modification	Eg5 2263 siRNA sequence	SEQ ID NO:	% 2'OMe-Modified	% Modified in DS Region
U/U	5'-CUGAAGACCUGAAGACAAUdTdT-3' 3'-dTdTGACUUCUGGACUUCUGUUA-5'	1 2	6/42 = 14.3%	6/38 = 15.8%

Column 1: "U/U" = 2'OMe-uridine modified siRNA duplex; Column 2: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. "dT" = deoxythymidine. Column 3: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 4: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

[0344] The lipid components and physical characteristics of the SNALP formulations are summarized in Table 2. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 2. Characteristics of the SNALP formulations used in this study.

Sample No.	Formulation Composition, Mole % PEG(2000)-C-DMA I DLinDMA I DPPC I Cholesterol	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
1	2 I 40 I 10 I 48	12.4	57	0.07	90
2	1.8 I 36.4 I 18.2 I 43.6	14.0	72	0.12	89
3	1.4 I 27.0 I 6.8 I 64.9	16.5	70	0.12	92
4	1.3 I 25.3 I 12.7 I 60.8	18.1	76	0.07	93
5	3.9 I 39.2 I 9.8 I 47.1	13.5	53	0.27	86
6	3.6 I 35.7 I 17.9 I 42.9	15.1	58	0.18	87
7	2.7 I 26.7 I 6.7 I 64.0	17.6	56	0.17	92
8	2.5 I 25.0 I 12.5 I 60.0	19.2	61	0.13	92
9	1.4 I 57.1 I 7.1 I 34.3	17.8	84	0.10	88
10	1.3 I 53.3 I 13.3 I 32.0	19.5	83	0.10	89
11	1.1 I 42.6 I 5.3 I 51.1	22.0	80	0.10	93
12	1.0 I 40.4 I 10.1 I 48.5	23.6	78	0.11	88
13	2.8 I 58.3 I 7.0 I 33.8	19.0	62	0.14	80
14	2.6 I 52.6 I 13.2 I 31.6	20.6	66	0.14	82
15	2.1 I 42.1 I 5.3 I 50.5	23.1	71	0.16	91
16	2 I 40 I 10 I 48	24.7	67	0.14	92

[0345] Silencing of Eg5 by siRNA transfection causes mitotic arrest and apoptosis in mammalian cells. Cell viability following transfection with SNALP containing an siRNA targeting Eg5 therefore provides a simple biological readout of *in vitro* transfection efficiency. Cell viability of *in vitro* cell cultures was assessed using the commercial reagent CellTiter-Blue® (Promega Corp.; Madison, WI), a resazurin dye that is reduced by metabolically active cells to the flourogenic product resorufin. The human colon cancer cell

line HT29 was cultured using standard tissue culture techniques. 72 hours after SNALP application, CellTiter-Blue® reagent was added to the culture to quantify the metabolic activity of the cells, which is a measure of cell viability. Data are presented as a percent of cell viability relative to ("untreated") control cells that received phosphate buffered saline (PBS) vehicle only.

[0346] Figure 1 shows that the 1:57 SNALP formulation containing Eg5 2263 U/U siRNA was among the most potent inhibitors of tumor cell growth at all siRNA concentrations tested (*see,* Figure 1B, Sample 9).

Example 3. ApoB siRNA Formulated as 1:57 SNALP Have Potent Silencing Activity *in vivo*.

[0347] SNALP formulations were prepared with an siRNA targeting apolipoprotein B (ApoB) as the nucleic acid component. ApoB is the main apolipoprotein of chylomicrons and low density lipoproteins (LDL). Mutations in ApoB are associated with hypercholesterolemia. ApoB occurs in the plasma in 2 main forms, ApoB48 and ApoB100, which are synthesized in the intestine and liver, respectively, due to an organ-specific stop codon. The ApoB siRNA used in this study is provided in Table 3. The modifications involved introducing 2'OMe-uridine or 2'OMe-guanosine at selected positions in the sense and antisense strands of the ApoB siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 3. siRNA duplex comprising sense and antisense ApoB RNA polynucleotides.

Position	Modification	ApoB siRNA sequence	SEQ ID NO:	% 2'OMe-Modified	% Modified in DS Region
I0048	U2/2 G1/2	5'-AGU**U**CA**U**CACAC**U**GAAUACC-3' 3'-GU**U**CACAGUAGU**U**G**A**C**U**UAU-5'	3 4	7/42 = 16.7%	7/38 = 18.4%

Column 1: The number refers to the nucleotide position of the 5' base of the sense strand relative to the mouse ApoB mRNA sequence XM_137955. Column 2: The numbers refer to the distribution of 2'OMe chemical modifications in each strand. Column 3: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. Column 4: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 5: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

[0348] The lipid components and physical characteristics of the formulations are summarized in Table 4. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments

Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 4. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition Lipid Name & Mole %	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2 | 40 | 10 | 48	12.4	59	0.15	93
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 2.2 | 44.4 | 53.3	10.7	55	0.17	91
4	PEG(2000)-C-DMA | DLinDMA | DOPC | Cholesterol 2 | 40 | 10 | 48	12.5	59	0.16	92
5	PEG(2000)-C-DMA | DLinDMA | DMPC | Cholesterol 2 | 40 | 10 | 48	12.2	56	0.11	92
6	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.8 | 36.4 | 18.2 | 43.6	13.8	66	0.16	93
7	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 2 | 40 | 10 | 48	12.4	56	0.12	92
8	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 27.0 | 6.8 | 64.9	16.5	60	0.10	93
9	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.3 | 25.3 | 12.7 | 60.8	18.1	74	0.13	92
10	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2.5 | 25.0 | 12.5 | 60.0	19.2	60	0.13	93
11	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.4 | 34.3	17.8	79	0.09	94
12	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.0 | 40.4 | 10.1 | 48.5	23.6	72	0.11	93
13	PEG(2000)-C-DMA | DLinDMA | DPPC 2 | 70 | 28	8.7	73	0.09	87
14	PEG(2000)-C-DMA | DLinDMA | DPPC 1.6 | 54.7 | 43.8	11.3	65	0.11	87

[0349] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 1 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0350] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0351] Figure 2 shows that the 1:57 SNALP formulation containing ApoB 10048 U2/2 G1/2 siRNA was the most potent at reducing ApoB expression *in vivo* (*see,* Group 11).

Example 4. ApoB siRNA Formulated as 1:57 SNALP Have Potent Silencing Activity *in vivo*.

[0352] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 5. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 5. Characteristics of the SNALP formulations used in this study.

SNALP (L:D ratio)	siRNA Payload	Particle Size (Polydispersity)	% Encapsulation
2:30 (13)	ApoB-10048 U2/2 G1/2	65 nm (0.16)	88
1:57 (9)	ApoB-10048 U2/2 G1/2	74 nm (0.10)	89

[0353] The 2:30 SNALP formulation used in this study is lipid composition 2:30:20:48 as described in molar percentages of PEG-C-DMA, DLinDMA, DSPC, and cholesterol (in that order). This formulation was prepared by syringe press at an input lipid to drug (L:D) ratio (mg:mg) of 13:1.

[0354] The 1:57 SNALP formulation used in this study is lipid composition 1.5:57.1:7:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation was prepared by syringe press at an input lipid to drug (L:D) ratio (mg:mg) of 9:1.

[0355] BALB/c mice (female, 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Days 0, 1, 2, 3 & 4 for a total of 5 doses per animal. Daily dosage was either 1.0 (for 2:30 SNALP) or 0.1 (for 1:57 SNALP) mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given IV injections of phosphate buffered saline (PBS) vehicle. On Study Day 7, 72 h after the last treatment, animals were euthanized and liver tissue was collected in RNAlater.

[0356] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0357] Figure 3 shows that the 1:57 SNALP containing ApoB 10048 U2/2 G1/2 siRNA was more than 10 times as efficacious as the 2:30 SNALP in mediating ApoB gene silencing in mouse liver at a 10-fold lower dose.

Example 5. ApoB siRNA Formulated as 1:57 or 1:62 SNALP Have Potent Silencing Activity *in vivo*.

[0358] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 6. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 6. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition Lipid Name & Mole %	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	76	0.06	89
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 1.5 | 61.5 | 36.9	8.1	76	0.04	86
4	PEG(2000)-C-DMA | DODMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.0	72	0.05	95
5	PEG(5000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.6	52	0.16	89
6	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 1.4 | 57.1 | 7.1 | 34.3	8.9	68	0.10	94
7	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	72	0.07	95
8	PEG(2000)-C-DMA | DLinDMA | DPPC 1.8 | 70.2 | 28.1	8.6	74	0.13	86

[0359] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 0.75 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0360] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0361] Figure 4 shows that the 1:57 and 1:62 SNALP formulations had comparable ApoB silencing activity *in vivo* (*see, e.g.,* Groups 2 & 3).

Example 6. ApoB siRNA Formulated as 1:62 SNALP Have Potent Silencing Activity *in vivo*.

[0362] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 7. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 7. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition, Mole % PEG(2000)-C-DMA I DLinDMA I Cholesterol	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	1.5 I 61.5 I 36.9	6.1	80	0.07	92
3	1.4 I 54.8 I 43.8	6.6	74	0.05	89
4	2.0 I 61.2 I 36.7	6.2	71	0.11	91
5	1.8 I 54.5 I 43.6	6.7	67	0.09	91
6	1.3 I 68.1 I 30.6	7.4	91	0.06	89
7	1.2 I 61.8 I 37.1	8.0	87	0.10	90
8	1.7 I 67.8 I 30.5	7.6	81	0.07	91
9	1.4 I 56.3 I 42.3	8.6	75	0.11	92
10	1.9 I 61.3 I 36.8	8.2	72	0.10	91
11	1.8 I 56.1 I 42.1	8.8	70	0.10	90
12	1.3 I 66.7 I 32.0	9.5	89	0.09	89
13	1.2 I 61.7 I 37.0	10.0	87	0.10	91
14	1.7 I 66.4 I 31.9	9.6	82	0.11	90
15	1.5 I 61.5 I 36.9	10.1	79	0.10	91

[0363] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 0.1 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0364] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene

assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0365] Figure 5 shows that the 1:62 SNALP formulation was one of the most potent inhibitors of ApoB expression at two different lipid:drug ratios (*i.e.*, 6.1 & 10.1) among the phospholipid-free SNALP formulations tested (*see*, Groups 2 & 15).

Example 7. *In vivo* Silencing of ApoB Expression Using 1:57 SNALP Prepared Via a Syringe Press or Gear Pump Process.

[0366] This study illustrates a comparison of the tolerability and efficacy of the 1:57 SNALP formulation with ApoB-targeting siRNA as prepared by various manufacturing processes. In particular, 1:57 SNALP was prepared by a syringe press or gear pump process using either PBS or citrate buffer (post-blend dilution) and administered intravenously in mice.

Experimental Design

[0367] Animal Model: Female BALB/c mice, 5 wks old, n=4 per group/cage.

[0368] siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

Tolerability:

Group	Formulation	IV Injection	
		siRNA mg/kg	Lipid mg/kg
1	PBS vehicle	Standard 10 mL/kg volume	
2	1|57 Citrate Direct Dil, Syringe Press	7	77
3	1|57 PBS Direct Dil, Syringe Press	7	96
4	1|57 PBS Direct Dil, Gear Pump	7	79
5	1|57 Citrate Direct Dil, Syringe Press	9	99
6	1|57 PBS Direct Dil, Syringe Press	9	123
7	1|57 PBS Direct Dil, Gear Pump	9	102

Efficacy:

Group	Formulation	IV Injection	
		siRNA mg/kg	Lipid mg/kg
8	PBS vehicle	Standard 10 mL/kg volume	
9	1|57 PBS Direct Dil, Syringe Press	0.05	0.68
10	1|57 PBS Direct Dil, Gear Pump	0.05	0.57
11	1|57 PBS Direct Dil, Syringe Press	0.1	1.36
12	1|57 PBS Direct Dil, Gear Pump	0.1	1.13

Formulation:

[0369] Formulations are provided at 0.005 to 0.9 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

[0370] Formulation Details:

1. Lipid composition "1|57 Citrate blend" used in this study is 1.4:57.1:7.1:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation has an input lipid to drug ratio of 8.9.
2. Gear pump set up included 0.8 mm T-connector and 400 mL/min speed.
3. siRNA used in this study is apoB-10048 U2/2 G1/2 siRNA.

[0371] Formulation Summary:

		Particle Size			Final L:D
	1:57 (9:1) + DOW siRNA	Zavg (nm)	Poly	% Encap	(mg:mg)
322-050807-1	Syringe PBS Blend	79	0.12	92	13.6
322-050807-2	Syringe Citrate Blend	86	0.11	91	11.0
322-050807-3	Gear PBS Blend	80	0.09	93	11.3

Procedures

[0372] **Treatment:** Just prior to the first treatment, animals are weighed and dose amounts are calculated based on the weight of individual animals (equivalent to 10 mL/kg, rounded to the nearest 10 μl). Test article is administered by IV injection through the tail vein once on Day 0 (1 dose total per animal). Body weight is measured daily (every 24 h) for the duration of the study. Cage-side observations are taken daily in concert with body weight measurements and additionally as warranted.

[0373] **Group 1-7 Endpoint:** Animals are sacrificed on Day 1, 24 h after test article administration. Blood is collected by cardiac puncture upon sacrifice. Whole amount is collected into a SST microtainer for serum. Clot for 30 (to 60) min at room temp., centrifuge for 5 min at 16,000xg & 16°C, invert to confirm centrifugation is complete, and store at 4°C. Analyze complete small-animal clinical chemistry panel plus AST and SDH. Top priority list: ALT, AST, SDH, Bilirubin, Alkaline Phosphatase, GGT, BUN, CPK, Glucose. Secondary priority list: Creatinine, Albumin, Globulin, Total Protein.

[0374] **Group 8-12 Endpoint:** Animals are sacrificed on Day 2, 48 h after test article administration. Blood is collected by cardiac puncture and processed for plasma. Immediately centrifuge for 5 min at 16,000xg (at 16°C). Record any observations of unusual plasma appearance. Pipette off clear plasma supernatant into a clean microfuge tube and store at -80°C. The following tissues are removed and weighed separately: liver and spleen.

The bottom (unattached) half of the left liver lobe is detached and submerged in ≥ 5 volumes of RNAlater (< 0.3 g in 1.5 mL RNAlater in 2.0 mL tube), stored at least 16 hours at 4°C prior to analysis and long term storage at -20°C or -80°C for archival purposes. Formulations are expected to be well tolerated. Mice which exhibit signs of distress associated with the treatment are terminated at the discretion of the vivarium staff.

[0375] Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

[0376] Data Analysis: Tolerability of treatment regime is monitored by animal appearance and behavior as well as body weight. Blood clinical chemistry is measured by automated analyzer. ApoB and GAPDH mRNA levels in liver are measured via QG assay. ApoB protein in plasma is measured via ELISA. Total cholesterol in plasma is measured via standard enzymatic/colorimetric assay.

Results

[0377] There was no body weight loss or change in animal appearance/behavior upon administration of the 1:57 SNALP formulations. Figure 6 shows that the tolerability of SNALP prepared by citrate buffer versus PBS direct dilution did not differ significantly in terms of blood clinical chemistry parameters. There was a tolerability difference between syringe citrate and syringe PBS at constant siRNA dosage, but that was likely an artifact dependent on the different final lipid:drug (L:D) ratios of these two preparations.

[0378] Figure 7 shows that the efficacy of the 1:57 SNALP prepared by gear pump was similar to the same SNALP prepared by syringe press. The tolerability profile was improved with the gear pump process, which could be attributed to increased initial encapsulation rate and decreased final L:D ratio.

Example 8. *In vivo* Silencing of ApoB Expression Using 1:57 SNALP Prepared Via a Direct Dilution or In-Line Dilution Process.

[0379] This study illustrates a comparison of the tolerability and efficacy of the 1:57 SNALP formulation with ApoB-targeting siRNA as prepared by a direct dilution or in-line dilution process at an input lipid to drug ratio of 6:1 or 9:1.

Experimental Design

[0380] Animal Model: Female BALB/c mice, 7 wks old.

[0381] siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

CBC/Diff:

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
1	3	PBS	-	-
2	3	1|57 SNALP (9:1)	7 mg/kg	71 mg/kg
3	3	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg

Clinical Chemistry:

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
4	4	PBS	-	-
5	4	1|57 SNALP (9:1)	9 mg/kg	92 mg/kg
6	4	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg
7	4	(6:1) New 1|57 SNALP	11 mg/kg	78 mg/kg
8	4	(6:1) New 1|57 SNALP	13 mg/kg	93 mg/kg
9	4	(6:1) New 1|57 SNALP	15 mg/kg	107 mg/kg
10	4	(6:1) New 1|57 SNALP	17 mg/kg	121 mg/kg
11	4	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg

Activity:

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
12	4	PBS	-	-
13	4	1|57 SNALP (9:1)	0.05 mg/kg	0.51 mg/kg
14	4	1|57 SNALP (9:1)	0.1 mg/kg	1.02 mg/kg
15	4	1|57 SNALP (9:1)	0.2 mg/kg	2.04 mg/kg
16	4	(6:1) New 1|57 SNALP	0.05 mg/kg	0.36 mg/kg
17	4	(6:1) New 1|57 SNALP	0.1 mg/kg	0.71 mg/kg
18	4	(6:1) New 1|57 SNALP	0.2 mg/kg	1.42 mg/kg
19	4	(6:1) New 1|57 SNALP	0.4 mg/kg	2.85 mg/kg

Formulation:

[0382] Formulations are provided at 0.005 to 1.7 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

[0383] Formulation Details:

1. "1|57 SNALP" used in this study is lipid composition 1.4:57.1:7.1:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation was prepared by gear pump at an input lipid to drug ratio of 9:1 (28 mM lipids) or 6:1 (14 mM lipids).
2. siRNA used in this study is apoB-10048 U2/2 G1/2 siRNA.

[0384] Formulation Summary:

	1|57 SNALP Gear PBS In-Line	Particle Size			Final L:D
		Zavg (nm)	Poly	% Encap	(mg:mg)
322-051407-1	Input 9:1	78	0.07	93	10.2
322-051407-2	Input 6:1	81	0.05	92	7.1

Procedures

[0385] Treatment: Just prior to the first treatment, animals are weighed and dose amounts are calculated based on the weight of individual animals (equivalent to 10 mL/kg, rounded to the nearest 10 μl). Test article is administered by IV injection through the tail vein once on Day 0 (1 dose total per animal). Body weight is measured daily (every 24 h) for the duration of the study. Cage-side observations are taken daily in concert with body weight measurements and additionally as warranted.

[0386] Endpoint: Animals are sacrificed on Day 1, 24 h after test article administration (Grps 1-10) or on Day 2, 48 h after test article administration (Grps 11-19).

[0387] Groups 1-3: Blood is collected by cardiac puncture upon sacrifice. Whole amount is collected into an EDTA microtainer, mixed immediately to prevent coagulation, and sent for analysis of CBC/Diff profile. Perform brief necropsy.

[0388] Groups 4-11: Blood is collected by cardiac puncture into a SST microtainer for serum. Clot for 30 (to 60) min at room temp., centrifuge for 5 min at 16,000xg & 16°C, invert to confirm centrifugation is complete, and store at 4°C. Analyze complete small-animal clinical chemistry panel plus AST and SDH. Top priority list: ALT, AST, SDH, Bilirubin, Alkaline Phosphatase, GGT, BUN, CPK, Glucose. Secondary priority list: Creatinine, Albumin, Globulin, Total Protein. Perform brief necropsy.

[0389] Groups 12-19: Blood is collected by cardiac puncture and processed for plasma: immediately centrifuge for 5 min at 16,000xg (at 16°C). Record any observations of unusual plasma appearance. Pipette off clear plasma supernatant into a clean microfuge tube and store at -80°C. The following tissues are removed: liver. The liver is not weighed; the bottom (unattached) half of the left liver lobe is detached and submerged in ≥ 5 volumes of RNAlater (< 0.3 g in 1.5 mL RNAlater in 2.0 mL tube), stored at least 16 hours at 4°C prior to analysis and long term storage at –80°C. Formulations are expected to be well tolerated. Mice which exhibit signs of distress associated with the treatment are terminated at the discretion of the vivarium staff.

[0390] Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

[0391] Data Analysis: Tolerability of treatment regime is monitored by animal appearance and behavior, and body weight. Blood clinical chemistry and CBC/Diff profile is measured

by automated analyzer. Liver ApoB mRNA is measured using the QuantiGene Assay. Plasma ApoB-100 is measured using ELISA. Plasma total cholesterol is measured using a standard enzymatic assay.

Results

Tolerability:

[0392] Figure 8 shows that there was very little effect on body weight 24 hours after 1:57 SNALP administration. The maximum weight loss of 3.6 ± 0.7% was observed at the highest drug dose of 17 mg/kg. There was also no obvious change in animal appearance/behavior at any of the dosages tested.

[0393] Figure 9 shows that there were no obvious changes in platelet count. Reduction of platelets can cause the mean platelet volume to increase as the body produces new platelets in compensation for the treatment-related decrease. Under the conditions of this study, the mean platelet volume did not change in SNALP-treated groups.

[0394] Figure 10 shows that clinically significant liver enzyme elevations (3xULN) occurred at drug dosages of 11 mg/kg for 1:57 SNALP at a lipid:drug (L:D) ratio of 10, and at 13 mg/kg at a L:D of 7. A slight dose response trend upwards in plasma total protein and globulin was also observed.

Efficacy:

[0395] Figure 11 shows that based on the liver mRNA QuantiGene analysis, the potency of the lower L:D SNALP was as good as that of the higher L:D SNALP at the tested drug dosages. In fact, the ApoB silencing activity was identical at the 0.05 and 0.1 mg/kg dosages. As such, the potency of the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) was similar to the potency of the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1) at reducing ApoB expression.

[0396] Figure 12 shows that ApoB protein and total cholesterol levels were reduced to a similar extent by the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) and the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1).

Therapeutic Index:

[0397] This study demonstrates that both the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) and the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1) caused about 60% ApoB liver mRNA silencing with a drug dose of 0.1 mg/kg. Interpolating from the available data points in Figure 10, a 10:1 final L:D ratio at 10 mg/kg may cause a similar

degree of enzyme elevation as a 7:1 final L:D ratio at 13 mg/kg. Using these activity and toxicity points, the therapeutic index for the 1:57 SNALP at a 10:1 final L:D ratio is (10 mg/kg) / (0.1 mg/kg) = 100 and the therapeutic index for the 1:57 SNALP at a 7:1 final L:D ratio is (13 mg/kg) / (0.1 mg/kg) = 130. Using this dataset, the therapeutic index for the 1:57 SNALP at a 7:1 final L:D ratio is 30% greater than the therapeutic index for the 1:57 SNALP at a 10:1 final L:D ratio.

Example 9. ***In vivo*** **Silencing of PLK-1 Expression Using 1:57 SNALP Increases Survival of Hep3B Tumor-Bearing Mice.**

[0398] SNALP containing polo-like kinase 1 (PLK-1) siRNA (1:57 SNALP formulation: 1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol) were tested for their effects on the survival of CD1 nu/nu mice bearing Hep3B liver tumors. PLK-1 is a serine/threonine kinase containing two functional domains: (1) a kinase domain; and (2) a polo-box domain (*see, e.g.,* Barr *et al.*, *Nat. Rev. Mol. Cell Biol.*, 5:429-440 (2004)). The activity and cellular concentration of PLK-1 are crucial for the precise regulation of cell division. PLK-1 is overexpressed in many cancer types including hepatoma and colon cancer, and PLK-1 expression often correlates with poor patient prognosis. Overexpression of PLK-1 (wild-type or kinase inactive) results in multinucleation (genetic instability). Hyperactive PLK-1 overrides the DNA damage checkpoint. Constitutive PLK-1 expression causes transformation of NIH 3T3 cells. PLK-1 phosphorylates the p53 tumor suppressor, thereby inhibiting the pro-apoptotic effects of p53. The PLK-1 siRNA used in this study are provided in Table 8. The modifications involved introducing 2'OMe-uridine or 2'OMe-guanosine at selected positions in the sense and antisense strands of the PLK-1 siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 8. siRNA duplexes comprising sense and antisense PLK-1 RNA polynucleotides.

siRNA	PLK-1 siRNA Sequence	SEQ ID NO:	% Modified in DS Region
PLK1424 U4/GU	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3' ~~(SEQ ID NO.57)~~ 3'-NNUC**U**AGUGGGAGG**A**AUUUAU-5' ~~(SEQ ID NO.54)~~	5 6	6/38 = 15.8%
PLK1424 U4/G	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3' ~~(SEQ ID NO.57)~~ 3'-NNUCUAGUGG**G**AG**G**AAUUUAU-5' ~~(SEQ ID NO.56)~~	5 7	7/38 = 18.4%

Column 1: The number after "PLK" refers to the nucleotide position of the 5' base of the sense strand relative to the start codon (ATG) of the human PLK-1 mRNA sequence NM_005030. Column 2: 2'-O-methyl (2'OMe) nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. N = deoxythymidine (dT) nucleotide, uridine (U) ribonucleotide, or

ribonucleotide having complementarity to the target sequence (antisense strand) or the complementary strand thereof (sense strand). Column 3: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

Experimental Groups

[0399] 20 CD1 nu/nu mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	SNALP dose	Sacrifice	Assay
A	20 to seed	I.H. $1.5x10^6$ Hep3B	Luc 1:57	9	Days 11, 14, 17, 21, 25, 28, 32, 35, 39, 42	10 x 2 mg/kg	When moribund	Survival Body Weights
B			PLK 1424 1:57	9				

Test Articles

[0400] All samples were filter-sterilized prior to dilution to working concentration. All tubes were labeled with the formulation date, lipid composition, and nucleic acid concentration. SNALP samples were provided at 0.2 mg/ml nucleic acid. A minimum of 20 ml of each SNALP was required to perform the study. Formulations for this study contained:

Group	Test Article Description
A	Luc U/U SNALP 1:57 (28mM lipid)
B	PLK1424 U4/GU SNALP 1:57 (28mM lipid) PLK1424 U4/G SNALP 1:57 (28mM lipid)

Procedures

Day 0 Mice will receive Anafen by SC injection (100 μg in 20 μl saline) immediately prior to surgery. Individual mice are anesthetized by isoflourane gas inhalation and eye lube applied to prevent excessive eye drying. While maintained under gas anesthesia from a nose cone, a single 1.5 cm incision across the midline will be made below the sternum. The left lateral hepatic lobe is then exteriorized using an autoclaved cotton wool bud. 25 μl of tumor cells suspended in PBS is injected into the lobe at a shallow angle using a leur tip Hamilton syringe (50 μl) and 30G (3/8") needle. Cells will be injected slowly (~ 30 s) and a swab applied to the puncture wound immediately after

needle withdrawal. After any bleeding has stopped (~1 min), the incision is closed with 5-6 sutures in the muscle wall and 3-4 skin clips. Cell suspensions will be thoroughly mixed immediately prior to each injection. Mice will recover from anesthesia in a clean cage lined with paper towel and monitored closely for 2-4 hours. Animals are then returned to normal housing.

Day 1 All mice will be lightly anesthetized by isoflourane gas and the sutures examined. Animals will then receive Anafen by SC injection (100 μg in 20 μl saline).

Day 10 Mice will be randomized into the appropriate treatment groups.

Day 11 **Groups A, B – Day 11:** All Animals will be administered SNALP at 2 mg/kg by IV injection via the lateral tail vein. Mice will be dosed according to body weight (10 ml/kg). Dosing will be repeated for 5 consecutive days based on initial weight.

Day 14-35 **Groups A, B – Days 14, 17, 21, 25, 28, 32, 35:** All Animals will be re-administered SNALP at 2 mg/kg by IV injection via the lateral tail vein. Mice will be dosed according to body weight (10 ml/kg).

Body weights Groups: Mice will be weighed on the day of dosing for 5 weeks, then twice weekly until close of the study.

Endpoint: Tumor burden and formulations are expected to be well tolerated. Mice that exhibit signs of distress associated with the treatment or tumor burden are terminated at the discretion of the vivarium staff.

Termination: Mice are anesthetized with a lethal dose of ketamine/xylazine followed by cervical dislocation.

Data Analysis: Survival and body weights are assayed.

Results

[0401] Figure 13 shows the mean body weights of mice during therapeutic dosing of PLK1424 SNALP in the Hep3B intrahepatic (I.H.) tumor model. The treatment regimen was well tolerated with no apparent signs of treatment-related toxicity.

[0402] Figure 14 shows that treatment with 1:57 SNALP-formulated PLK1424 caused a significant increase in the survival of Hep3B tumor-bearing mice. This *in vivo* anti-tumor effect was observed in the absence of any apparent toxicity or immune stimulation.

Example 10. *In vivo* Silencing of PLK-1 Expression Using 1:57 SNALP Induces Tumor Cell Apoptosis in Hep3B Tumor-Bearing Mice.

[0403] The objectives of this study were as follows:

1. To determine the level of mRNA silencing in established Hep3B liver tumors following a single IV administration of PLK1424 SNALP.
2. To confirm the mechanism of mRNA silencing by detecting specific RNA cleavage products using RACE-PCR.
3. To confirm induction of tumor cell apoptosis by histopathology.

[0404] The 1:57 SNALP formulation (1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol) was used for this study.

Experimental Groups

[0405] 20 SCID/beige mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	Sacrifice	Assay
A	20 to seed	I.H. $1x10^6$ Hep3B	PBS	6	1 x 2 mg/kg Day 20	24 h after treatment	Tumor QG
			Luc 1:57	7			Tumor RACE-PCR
B			PLK 1424 1:57	7			Histopathology
C							

Test Articles

[0406] All samples were filter-sterilized prior to dilution to working concentration. All tubes were labeled with the formulation date, lipid composition, and nucleic acid concentration. SNALP samples were provided at 0.2 mg/ml nucleic acid. A minimum of 2 ml of SNALP was required to perform the study. Formulations for this study contained:

Group	Test Article Description
A	PBS
B	Luc U/U 1:57 SNALP
C	PLK1424 U4/GU 1:57 SNALP

Procedures

Day 0 Mice will receive Anafen by SC injection (100 μg in 20 μl saline) immediately prior to surgery. Individual mice are anesthetized by isoflourane gas inhalation and eye lube applied to prevent excessive eye drying. While maintained under gas anesthesia from a nose cone, a single 1.5 cm incision across the midline will be made below the sternum. The left lateral hepatic lobe is then exteriorized using an autoclaved cotton wool bud. 25μl of tumor cells suspended in PBS is injected into the lobe at a shallow angle using a leur tip Hamilton syringe (50 μl) and 30G (3/8") needle. Cells will be injected slowly (~30 s) and a swab applied to the puncture wound immediately after needle withdrawal. After any bleeding has stopped (~1 min), the muscle wall incision is closed with 5-6 sutures. The skin incision is then closed with 3-4 metal skin clips. Cell suspensions will be thoroughly mixed immediately prior to each injection. Mice will recover from anesthesia in a clean cage lined with paper towel and monitored closely for 2-4 hours. Animals are then returned to normal housing.

Day 1 All mice will be lightly anesthetized by isoflourane gas and the sutures examined. Animals will then receive Anafen by SC injection (100 μg in 20 μl saline).

Day 7 Mice will be randomized into the appropriate treatment groups.

Day 20 **Groups A-C:** Mice will be weighed and then administered either PBS, Luc, or PLK1424 SNALP by IV injection via the lateral tail vein. SNALP will be dosed at 2 mg/kg or equivalent volume (10 ml/kg) according to body weight.

Day 21 **Groups A-C:** All mice will be weighed and then euthanized by lethal anesthesia.

Tumor bearing liver lobes from all mice in each group will be weighed and collected into RNALater for RNA analysis.

Endpoint: Tumor burden and formulations are expected to be well tolerated. Mice that exhibit signs of distress associated with the

treatment or tumor burden are terminated at the discretion of the vivarium staff.

Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine followed by cervical dislocation.

Data Analysis: mRNA analysis of liver tumors by bDNA (QG) assay and RACE-PCR.

Tumor cell apoptosis by histopathology.

Results

[0407] Body weights were monitored from Day 14 onwards to assess tumor progression. On Day 20, 6 mice showing greatest weight loss were randomized into each of the 3 groups and treated. All six mice had substantial-large I.H. tumors at sacrifice (Day 21). Treatment of the remaining 14 mice was therefore initiated on the Day 21 (sacrifice Day 22). 10/14 mice had substantial tumors; 2/14 mice had small/probable tumors; and 2/14 mice had no visible tumor burden.

[0408] Figure 15 shows data from Quantigene assays used to measure human (tumor)-specific PLK-1 mRNA levels. A single 2 mg/kg dose of 1:57 SNALP reduced PLK-1 mRNA levels by about 50% in intrahepatic Hep3B tumors growing in mice.

[0409] Figure 16 shows that a specific cleavage product of PLK-1 mRNA was detectable in mice treated with PLK1424 SNALP by 5' RACE-PCR. No specific PCR product was detectable in mice treated with either PBS or control (Luc) SNALP. Nucleotide sequencing of the PCR product confirmed the predicted cleavage site by PLK1424 siRNA-mediated RNA interference in the PLK-1 mRNA.

[0410] Figure 17 shows Hep3B tumor histology in mice treated with either Luc SNALP (top) or PLK1424 SNALP (bottom). Luc SNALP-treated mice displayed normal mitoses in Hep3B tumors, whereas PLK1424 SNALP-treated mice exhibited numerous aberrant mitoses and tumor cell apoptosis in Hep3B tumors.

Conclusion

[0411] This example illustrates that a single administration of PLK1424 1:57 SNALP to Hep3B tumor-bearing mice induced significant *in vivo* silencing of PLK-1 mRNA. This reduction in PLK-1 mRNA was confirmed to be mediated by RNA interference using 5' RACE-PCR analysis. Importantly, PLK-1 mRNA silencing by the 1:57 SNALP formulation profoundly disrupted tumor cell proliferation (mitosis), causing subsequent apoptosis of

tumor cells. As demonstrated in the previous example, this anti-tumor effect translated into extended survival times in the tumor-bearing mice.

Example 11. Comparison of 1:57 PLK-1 SNALP Containing Either PEG-cDMA or PEG-cDSA in a Subcutaneous Hep3B Tumor Model.

[0412] This example demonstrates the utility of the PEG-lipid PEG-cDSA (3-N-[(-Methoxypoly(ethylene glycol)2000)carbamoyl]-1,2-distearyloxypropylamine) in the 1:57 formulation for systemically targeting distal (*e.g.*, subcutaneous) tumors. In particular, this example compares the tumor targeting ability of 1:57 PLK-1 SNALPs containing either PEG-cDMA (C_{14}) or PEG-cDSA (C_{18}). Readouts are tumor growth inhibition and PLK1 mRNA silencing. The PLK-1 siRNA used was PLK1424 U4/GU, the sequence of which is provided in Table 8.

[0413] Subcutaneous (S.C.) Hep3B tumors were established in scid/beige mice. Multidose anti-tumor efficacy of 1:57 PLK-1 SNALP was evaluated for the following groups (n=5 for each group): (1) "Luc-cDMA" - PEG-cDMA Luc SNALP; (2) "PLK-cDMA" - PEG-cDMA PLK-1 SNALP; and (3) "PLK-cDSA" - PEG-cDSA PLK-1 SNALP. Administration of 6 x 2mg/kg siRNA was initiated once tumors reached about 5 mm in diameter (Day 10). Dosing was performed on Days 10, 12, 14, 17, 19, and 21. Tumors were measured by caliper twice weekly.

[0414] Figure 18 shows that multiple doses of 1:57 PLK-1 SNALP containing PEG-cDSA induced the regression of established Hep3B S.C. tumors. In particular, 5/5 tumors in the PLK1-cDSA treated mice appeared flat, measurable only by discoloration at the tumor site.

[0415] Figure 19 shows the mRNA silencing of 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration. The extent of silencing observed with the PLK1-cDSA SNALP correlated with the anti-tumor activity in the multi-dose study shown in Figure 18.

[0416] The Luc-cDMA SNALP-treated group, which had developed large S.C. tumors at Day 24, were then administered PLK-cDSA SNALP on Days 24, 26, 28, 31, 33, and 35. There was no additional dosing of the original PLK-1 SNALP-treated groups. The results from this crossover dosing study with large established tumors is provided in Figure 20, which shows that PLK1-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

[0417] A comparison of the effect of PEG-cDMA and PEG-cDSA 1:57 SNALPs on PLK-1 mRNA silencing was performed using established intrahepatic Hep3B tumors in scid/beige mice. A single 2 mg/kg dose of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-

cDSA was administered intravenously. Liver/tumor samples were collected at 24 and 96 hours after SNALP treatment. Control = 2 mg/kg Luc-cDMA SNALP at 24 hours.

[0418] Figure 21 shows that PLK-cDMA SNALP and PLK-cDSA SNALP had similar silencing activities after 24 hours, but that the PLK-cDSA SNALP may increase the duration of mRNA silencing in intrahepatic tumors.

[0419] Figure 22 shows the blood clearance profile of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA. The extended blood circulation times observed for the PLK-cDSA SNALP may enable the increased accumulation and activity at distal (*e.g.*, subcutaneous) tumor sites.

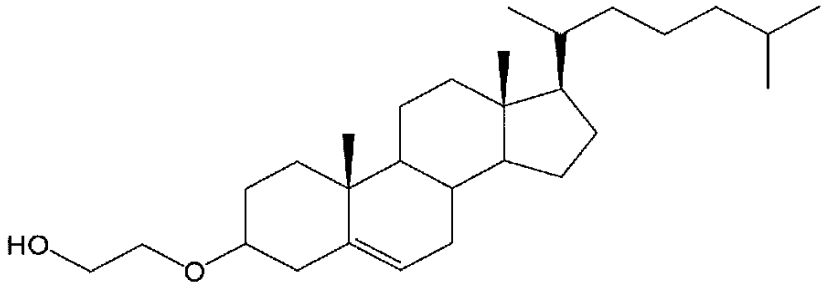
[0420] Thus, this study shows that the 1:57 PEG-cDSA SNALP formulation can be used to preferentially target tumors outside of the liver, whereas the 1:57 PEG-cDMA SNALP can be used to preferentially target the liver.

Example 12. Synthesis of Cholesteryl-2'-Hydroxyethyl Ether.

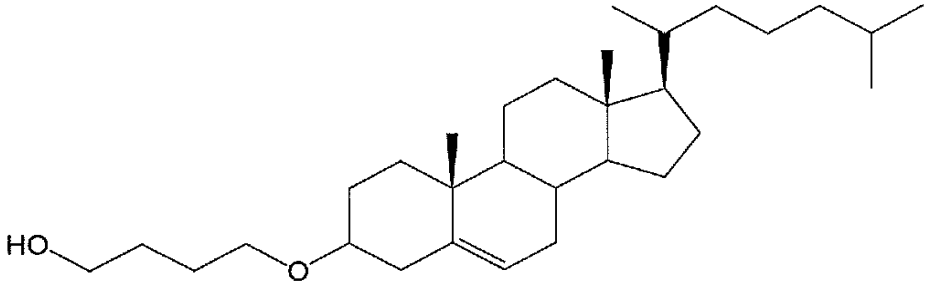
[0421] Step 1: A 250 ml round bottom flask containing cholesterol (5.0 g, 12.9 mmol) and a stir bar was sealed and flushed with nitrogen. Toluenesulphonyl chloride (5.0 g, 26.2 mmol) was weighed into a separate 100-mL round bottom flask, also sealed and flushed with nitrogen. Anhydrous pyridine (2 x 50 ml) was delivered to each flask. The toluenesulphonyl chloride solution was then transferred, via cannula, into the 250 ml flask, and the reaction stirred overnight. The pyridine was removed by rotovap, and methanol (80 ml) added to the residue. This was then stirred for 1 hour until a homogeneous suspension was obtained. The suspension was filtered, washed with acetonitrile (50 ml), and dried under vacuum to yield cholesteryl tosylate as a fluffy white solid (6.0 g, 86%).

[0422] Step 2: Cholesteryl tosylate (2.0 g, 3.7 mmol), 1,4-dioxane (50 mL), and ethylene glycol (4.6 g, 74 mmol) were added to a 100 ml flask containing a stir bar. The flask was fitted with a condenser, and refluxed overnight. The dioxane was then removed by rotovap, and the reaction mixture suspended in water (100 ml). The solution was transferred to a separating funnel and extracted with chloroform (3 x 100 ml). The organic phases were combined, washed with water (2 x 150 ml), dried over magnesium sulphate, and the solvent removed. The crude product was purified by column chromatography (5% acetone/hexane) to yield the product as a white solid (1.1 g, 69%).

[0423] The structures of the cholesterol derivatives cholesteryl-2'-hydroxyethyl ether and cholesteryl-4'-hydroxybutyl ether are as follows:



Cholesteryl-2'-hydroxyethyl ether



Cholesteryl-4'-hydroxybutyl ether

[0424] It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reading the above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. The disclosures of all articles and references, including patent applications, patents, PCT publications, and Genbank Accession Nos., are incorporated herein by reference for all purposes.

WHAT IS CLAIMED IS:

1. A nucleic acid-lipid particle comprising:

(a) a nucleic acid;

(b) a cationic lipid comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle;

(c) a non-cationic lipid comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and

(d) a conjugated lipid that inhibits aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

2. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid comprises a small interfering RNA (siRNA).

3. The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises from about 15 to about 60 nucleotides.

4. The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises at least one modified nucleotide.

5. The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises at least one 2'-O-methyl (2'OMe) nucleotide.

6. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), or a mixture thereof.

7. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA).

8. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle.

9. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from about 52 mol % to about 62 mol % of the total lipid present in the particle.

10. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises cholesterol or a derivative thereof.

11. The nucleic acid-lipid particle of claim 10, wherein the cholesterol or derivative thereof comprises from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle.

12. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises a phospholipid.

13. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises a mixture of a phospholipid and cholesterol or a derivative thereof.

14. The nucleic acid-lipid particle of claim 13, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

15. The nucleic acid-lipid particle of claim 13, wherein the phospholipid comprises from about 4 mol % to about 10 mol % of the total lipid present in the particle and the cholesterol comprises from about 30 mol % to about 40 mol % of the total lipid present in the particle.

16. The nucleic acid-lipid particle of claim 13, wherein the phospholipid comprises from about 10 mol % to about 30 mol % of the total lipid present in the particle and the cholesterol comprises from about 10 mol % to about 30 mol % of the total lipid present in the particle.

17. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

18. The nucleic acid-lipid particle of claim 17, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

19. The nucleic acid-lipid particle of claim 18, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

20. The nucleic acid-lipid particle of claim 19, wherein the PEG has an average molecular weight of about 2,000 daltons.

21. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises from about 1 mol % to about 2 mol % of the total lipid present in the particle.

22. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid in the nucleic acid-lipid particle is not substantially degraded after incubation of the particle in serum at 37°C for 30 minutes.

23. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

24. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid-lipid particle has a lipid:nucleic acid mass ratio of from about 5 to about 15.

25. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid-lipid particle has a median diameter of from about 40 nm to about 150 nm.

26. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 1 and a pharmaceutically acceptable carrier.

27. A nucleic acid-lipid particle comprising:

(a) an siRNA;

(b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle;

(c) cholesterol or a derivative thereof comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and

(d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle.

28. The nucleic acid-lipid particle of claim 27, wherein the cationic lipid comprises DLinDMA.

29. The nucleic acid-lipid particle of claim 27, wherein the cationic lipid comprises DLin-K-C2-DMA.

30. The nucleic acid-lipid particle of claim 27, wherein the PEG-lipid conjugate comprises a PEG-DAA conjugate.

31. The nucleic acid-lipid particle of claim 27, wherein the nucleic acid-lipid particle comprises about 61.5 mol % cationic lipid, about 36.9% cholesterol or a derivative thereof, and about 1.5 mol % PEG-lipid conjugate.

32. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 27 and a pharmaceutically acceptable carrier.

33. A nucleic acid-lipid particle, comprising:

(a) an siRNA;

(b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle;

(c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and

(d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle.

34. The nucleic acid-lipid particle of claim 33, wherein the cationic lipid comprises DLinDMA.

35. The nucleic acid-lipid particle of claim 33, wherein the cationic lipid comprises DLin-K-C2-DMA.

36. The nucleic acid-lipid particle of claim 33, wherein the phospholipid comprises DPPC.

37. The nucleic acid-lipid particle of claim 33, wherein the PEG-lipid conjugate comprises a PEG-DAA conjugate.

38. The nucleic acid-lipid particle of claim 33, wherein the nucleic acid-lipid particle comprises about 57.1 mol % cationic lipid, about 7.1 mol % phospholipid, about 34.3 mol % cholesterol or a derivative thereof, and about 1.4 mol % PEG-lipid conjugate.

39. The nucleic acid-lipid particle of claim 33, wherein the nucleic acid-lipid particle comprises about 57.1 mol % cationic lipid, about 20 mol % phospholipid, about 20 mol % cholesterol or a derivative thereof, and about 1.4 mol % PEG-lipid conjugate.

40. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 33 and a pharmaceutically acceptable carrier.

41. A method for introducing a nucleic acid into a cell, the method comprising:

contacting the cell with a nucleic acid-lipid particle of claim 1, 27, or 33.

42. The method of claim 41, wherein the cell is in a mammal.

43. A method for the *in vivo* delivery of a nucleic acid, the method comprising:

administering to a mammalian subject a nucleic acid-lipid particle of claim 1, 27, or 33.

44. The method of claim 43, wherein the administration is selected from the group consisting of oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal.

45. A method for treating a disease or disorder in a mammalian subject in need thereof, the method comprising:

administering to the mammalian subject a therapeutically effective amount of a nucleic acid-lipid particle of claim 1, 27, or 33.

46. The method of claim 45, wherein the disease or disorder is selected from the group consisting of a viral infection, a liver disease or disorder, and cancer.

ABSTRACT OF THE DISCLOSURE

The present invention provides novel, stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles. More particularly, the present invention provides stable nucleic acid-lipid particles (SNALP) comprising a nucleic acid (such as one or more interfering RNA), methods of making the SNALP, and methods of delivering and/or administering the SNALP.

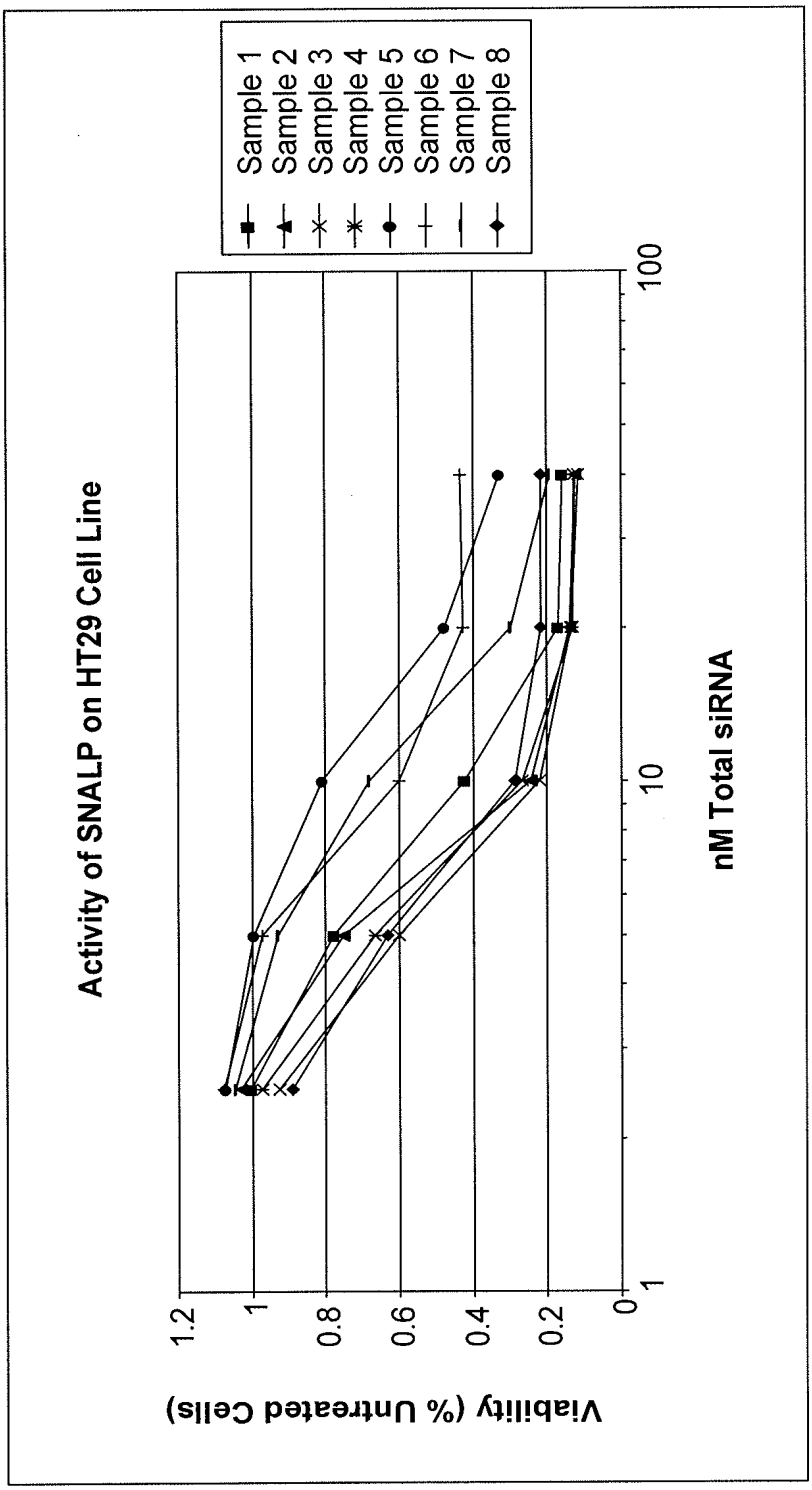

Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
nM Total siRNA
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Sample 7
Sample 8
0
0.2
0.4
0.6
0.8
1
1.2
1
10
100

FIG. 1A

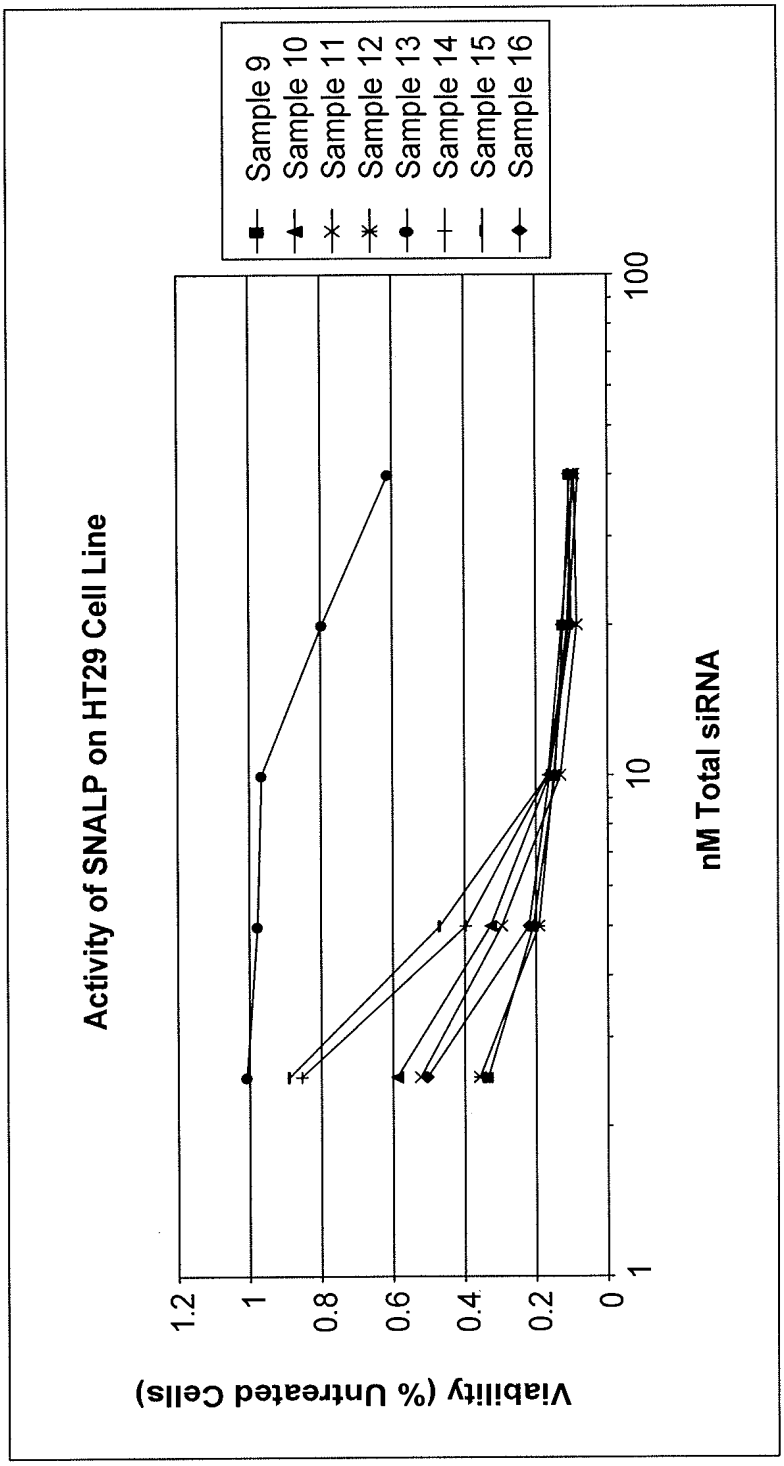

Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA
Sample 9
Sample 10
Sample 11
Sample 12
Sample 13
Sample 14
Sample 15
Sample 16

FIG. 1B

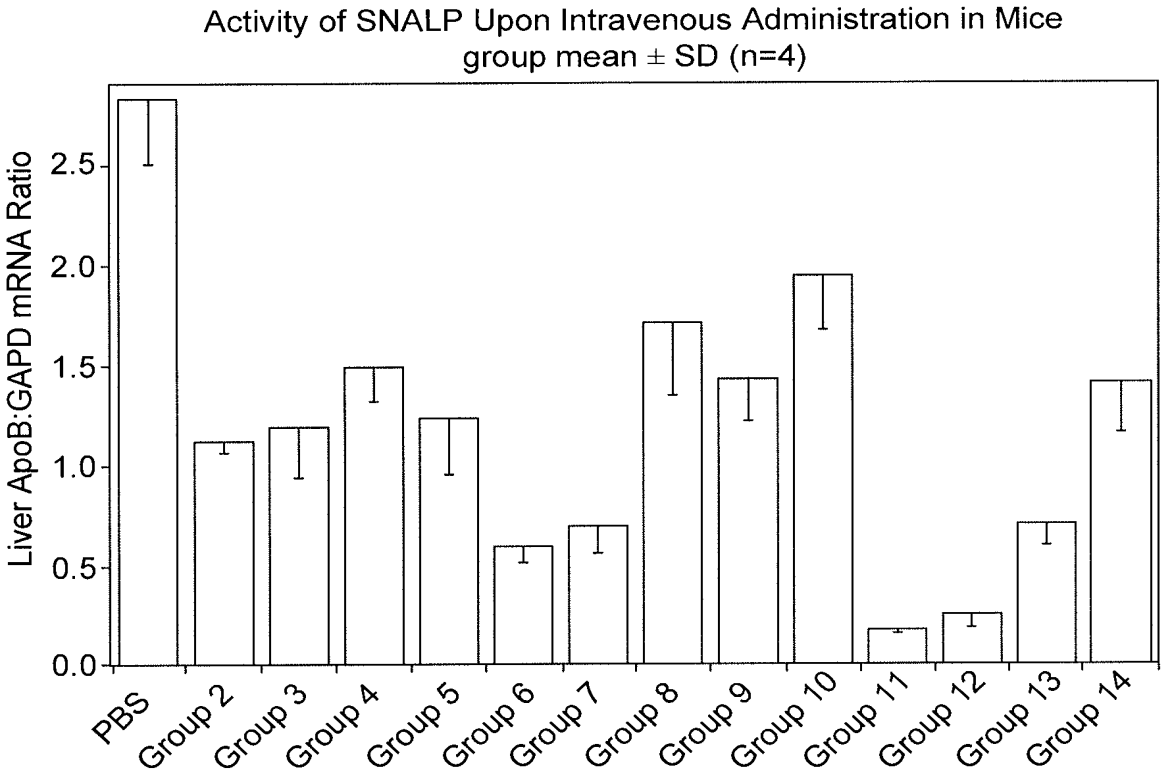

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
0.0
0.5
1.0
1.5
2.0
2.5
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14

FIG. 2

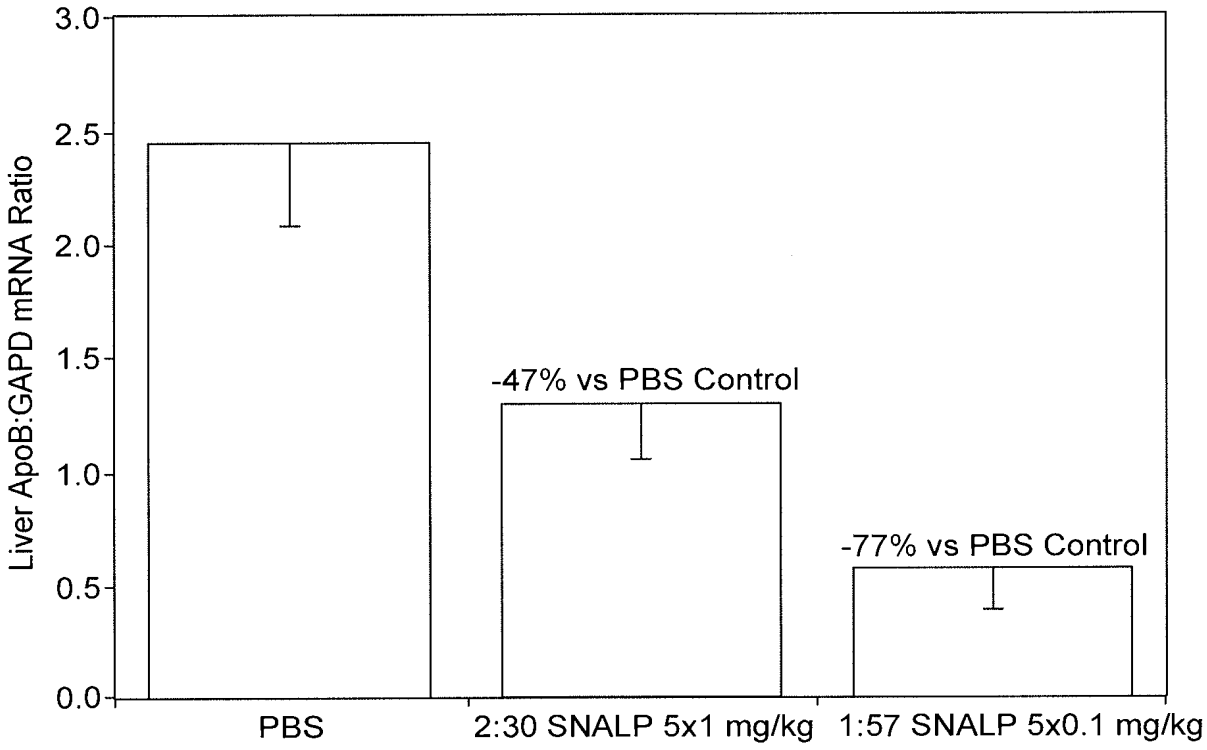

Liver ApoB:GAPD mRNA Ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-47% vs PBS Control
-77% vs PBS Control
PBS
2:30 SNALP 5x1 mg/kg
1:57 SNALP 5x0.1 mg/kg

FIG. 3

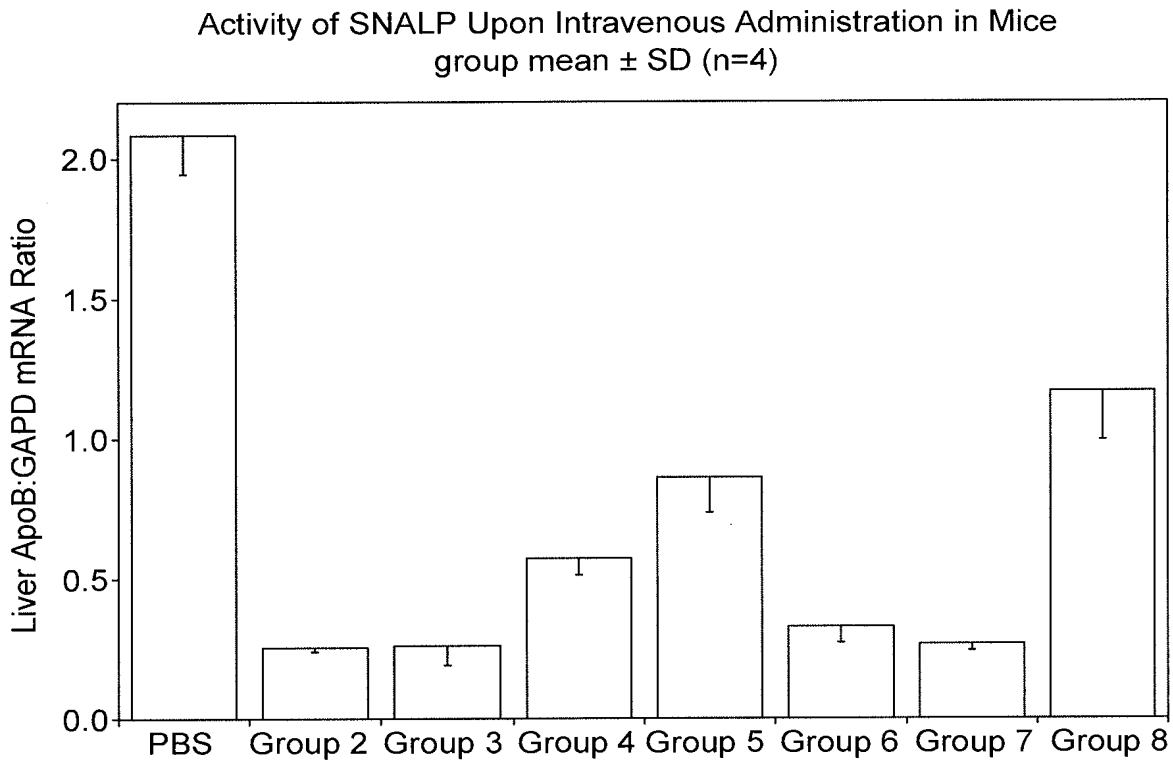

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8

FIG. 4

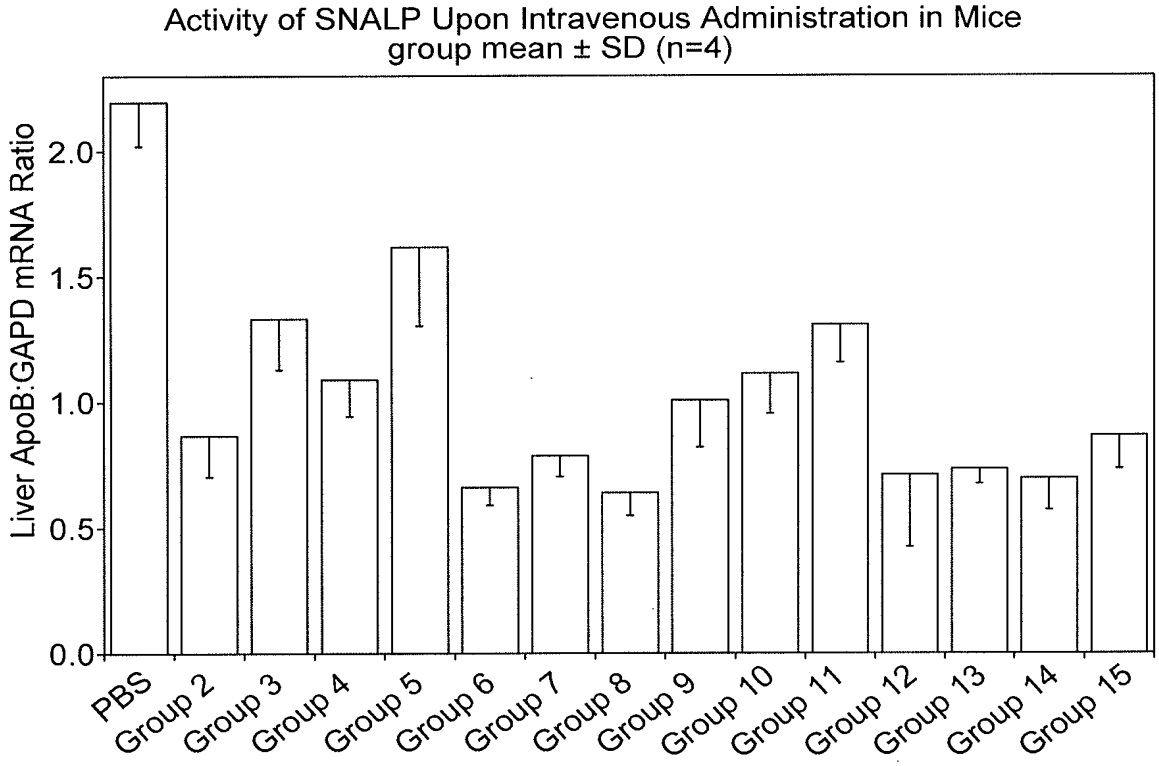

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14
Group 15

FIG. 5

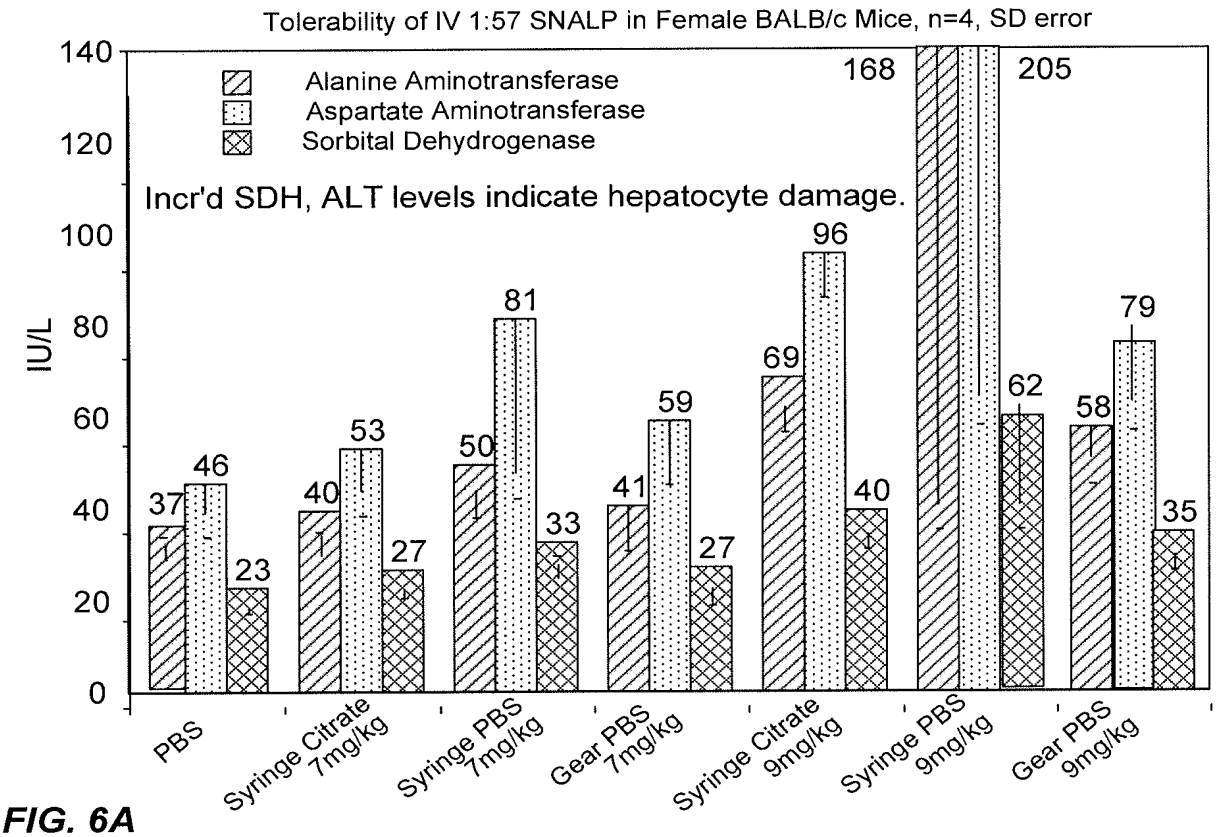

Tolerability of IV 1:57 SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Incr'd SDH, ALT levels indicate hepatocyte damage.
IU/L
0
20
40
60
80
100
120
140
37
46
23
40
53
27
50
81
33
41
59
27
69
96
40
168
205
62
58
79
35
PBS
Syringe Citrate 7mg/kg
Syringe PBS 7mg/kg
Gear PBS 7mg/kg
Syringe Citrate 9mg/kg
Syringe PBS 9mg/kg
Gear PBS 9mg/kg

FIG. 6A

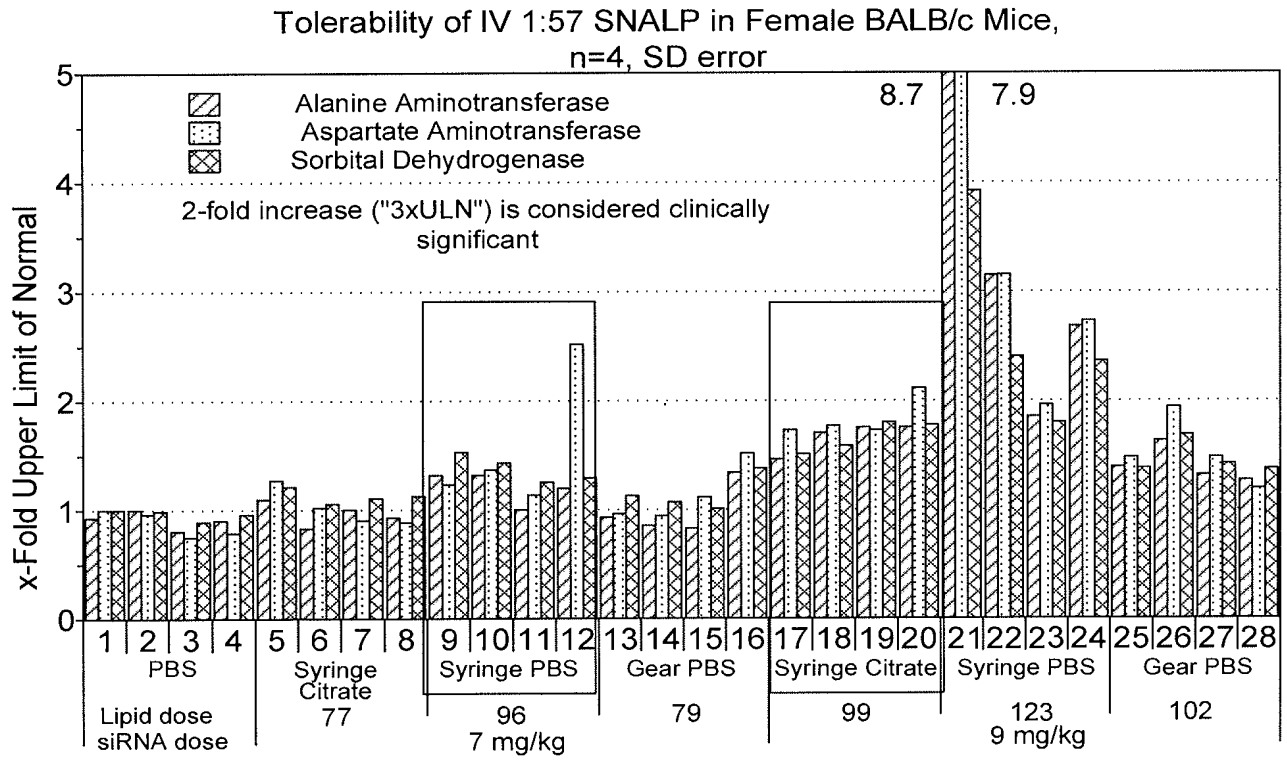

Tolerability of IV 1:57 SNALP in Female BALB/c Mice,
n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
0
1
2
3
4
5
8.7
7.9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
PBS
Syringe Citrate
Syringe PBS
Gear PBS
Syringe Citrate
Syringe PBS
Gear PBS
Lipid dose
siRNA dose
77
96
7 mg/kg
79
99
123
9 mg/kg
102

FIG. 6B

FIG. 7A

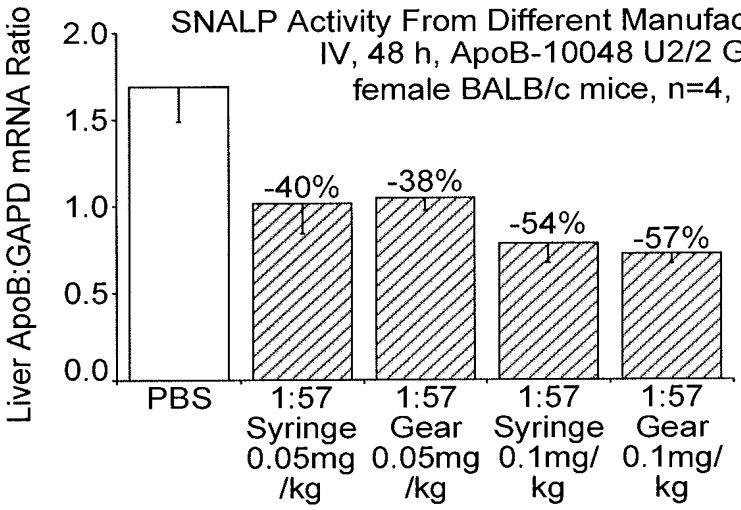


FIG. 7B

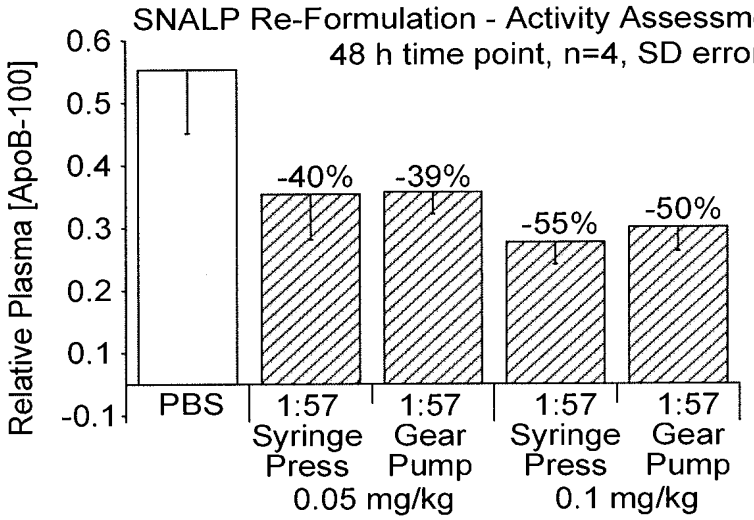
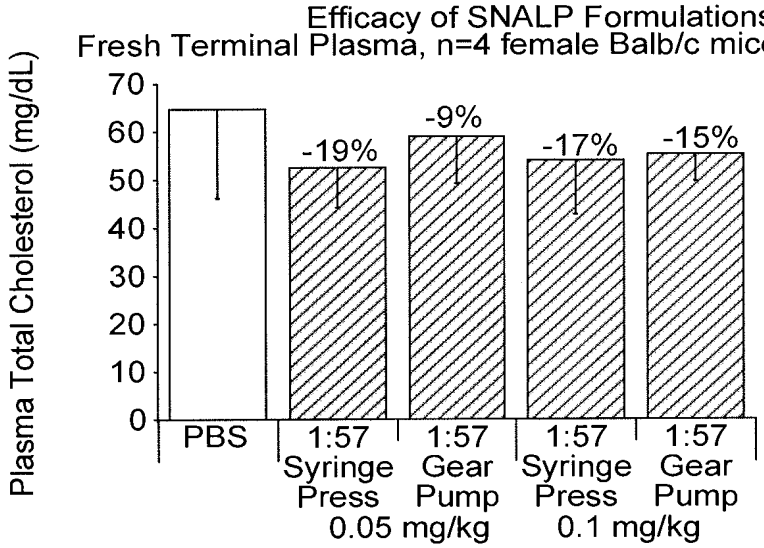


FIG. 7C



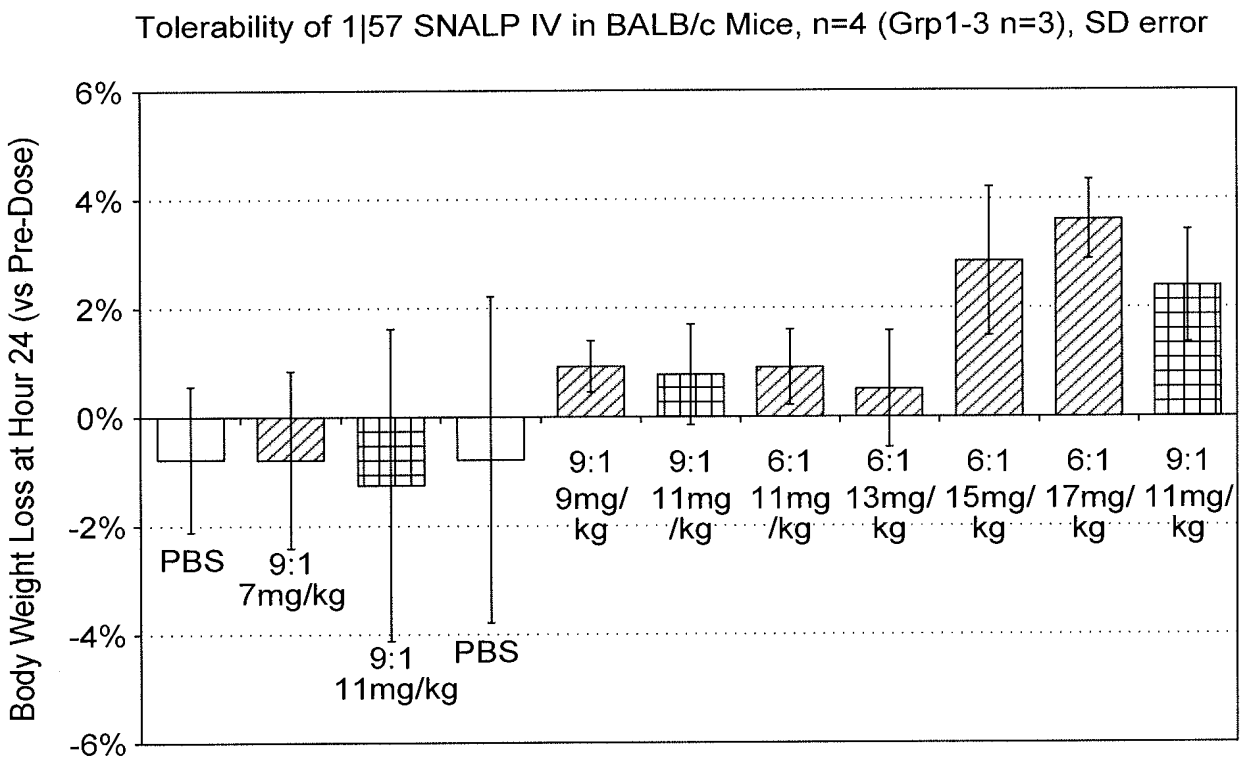

Tolerability of 1|57 SNALP IV in BALB/c Mice, n=4 (Grp1-3 n=3), SD error
Body Weight Loss at Hour 24 (vs Pre-Dose)
6%
4%
2%
0%
-2%
-4%
-6%
PBS
9:1 7mg/kg
9:1 11mg/kg
PBS
9:1 9mg/kg
9:1 11mg/kg
6:1 11mg/kg
6:1 13mg/kg
6:1 15mg/kg
6:1 17mg/kg
9:1 11mg/kg

FIG. 8

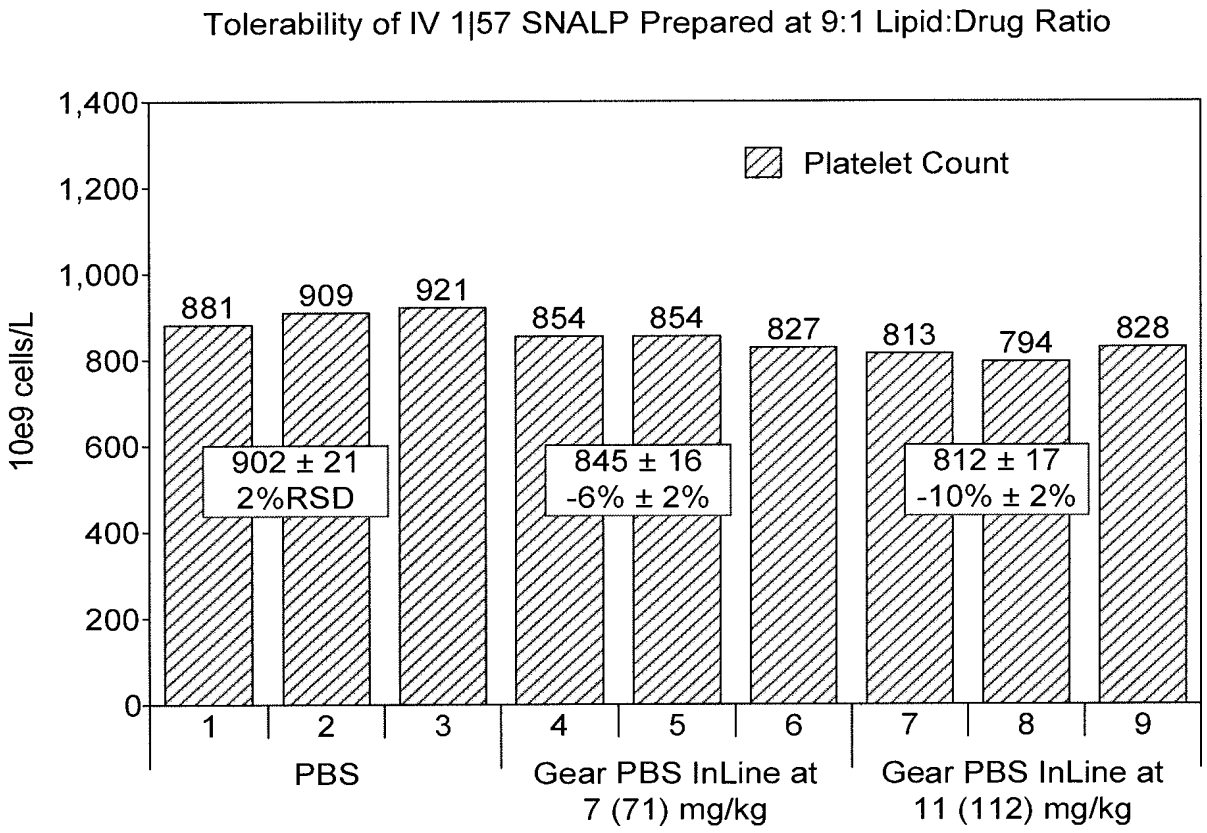

Tolerability of IV 1|57 SNALP Prepared at 9:1 Lipid:Drug Ratio
Platelet Count
10e9 cells/L
1,400
1,200
1,000
800
600
400
200
0
881
909
921
854
854
827
813
794
828
902 ± 21
2%RSD
845 ± 16
-6% ± 2%
812 ± 17
-10% ± 2%
1
2
3
4
5
6
7
8
9
PBS
Gear PBS InLine at
7 (71) mg/kg
Gear PBS InLine at
11 (112) mg/kg

FIG. 9

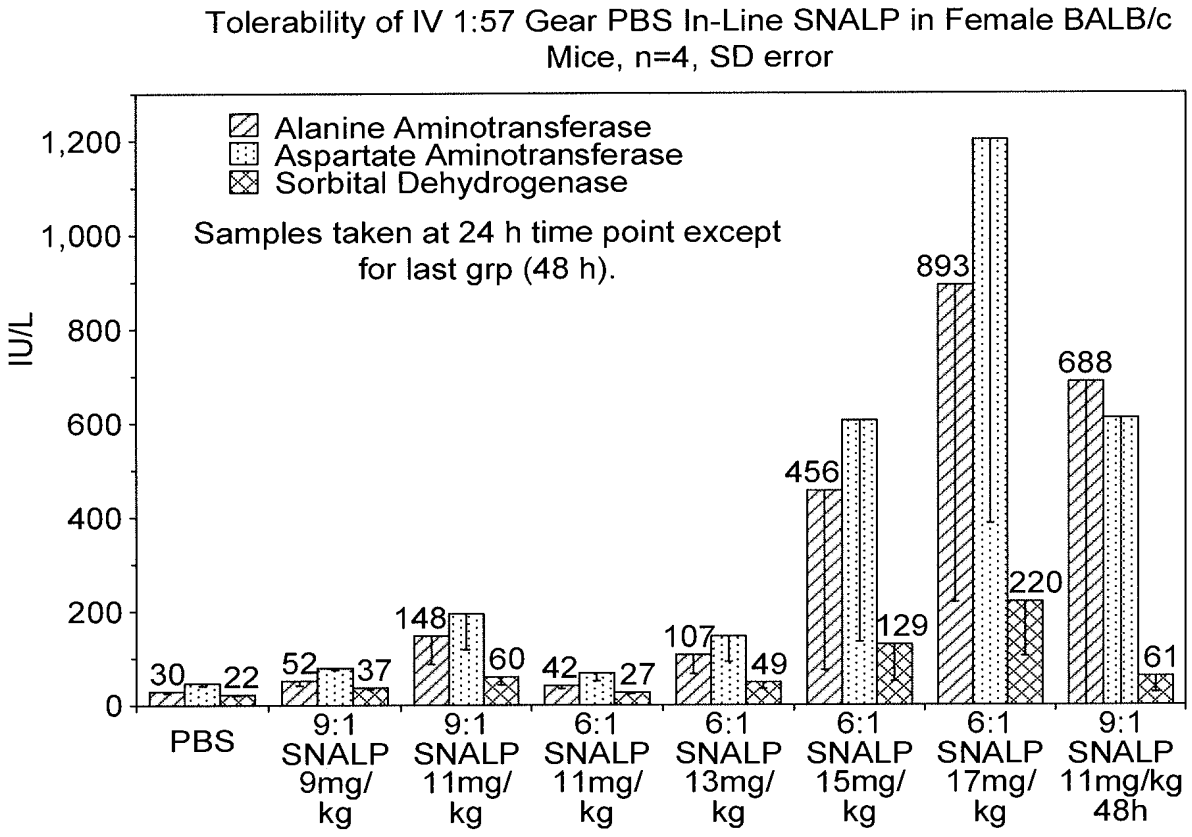

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Samples taken at 24 h time point except for last grp (48 h).
IU/L
1,200
1,000
800
600
400
200
0
30
22
52
37
148
60
42
27
107
49
456
129
893
220
688
61
PBS
9:1 SNALP 9mg/kg
9:1 SNALP 11mg/kg
6:1 SNALP 11mg/kg
6:1 SNALP 13mg/kg
6:1 SNALP 15mg/kg
6:1 SNALP 17mg/kg
9:1 SNALP 11mg/kg 48h

FIG. 10A

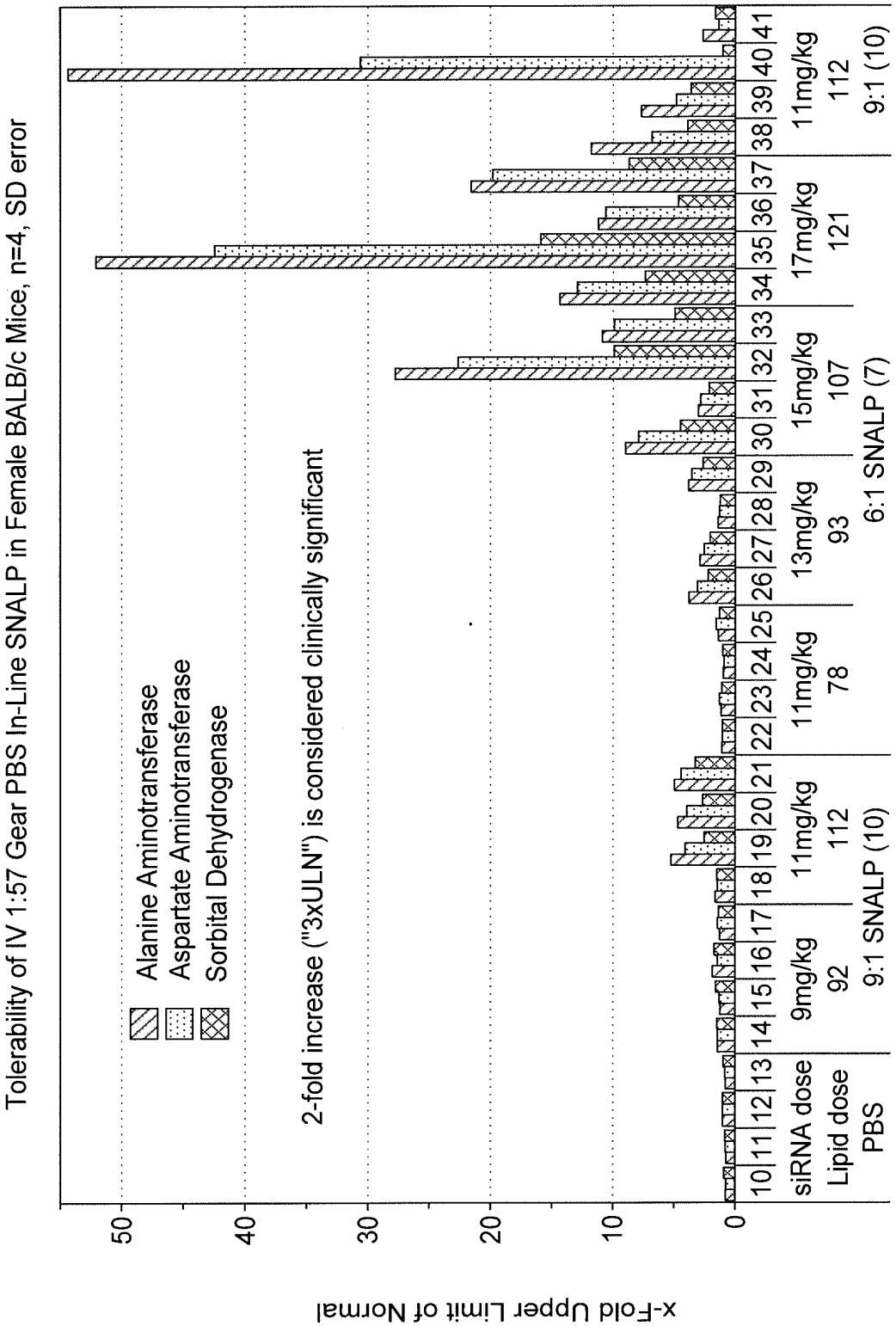

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
0
10
20
30
40
50
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41
siRNA dose
Lipid dose
PBS
9mg/kg
92
9:1 SNALP (10)
11mg/kg
112
11mg/kg
78
13mg/kg
93
15mg/kg
107
6:1 SNALP (7)
17mg/kg
121
11mg/kg
112
9:1 (10)

FIG. 10B

FIG. 11A

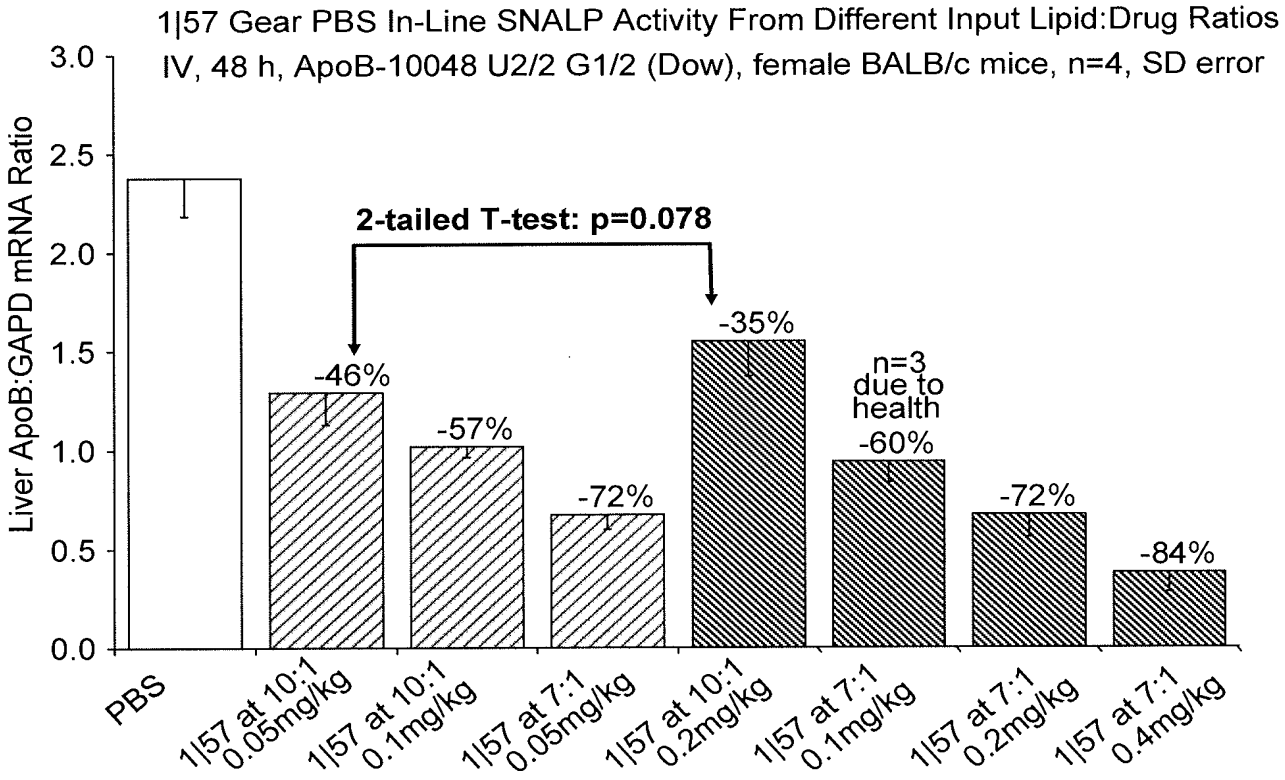
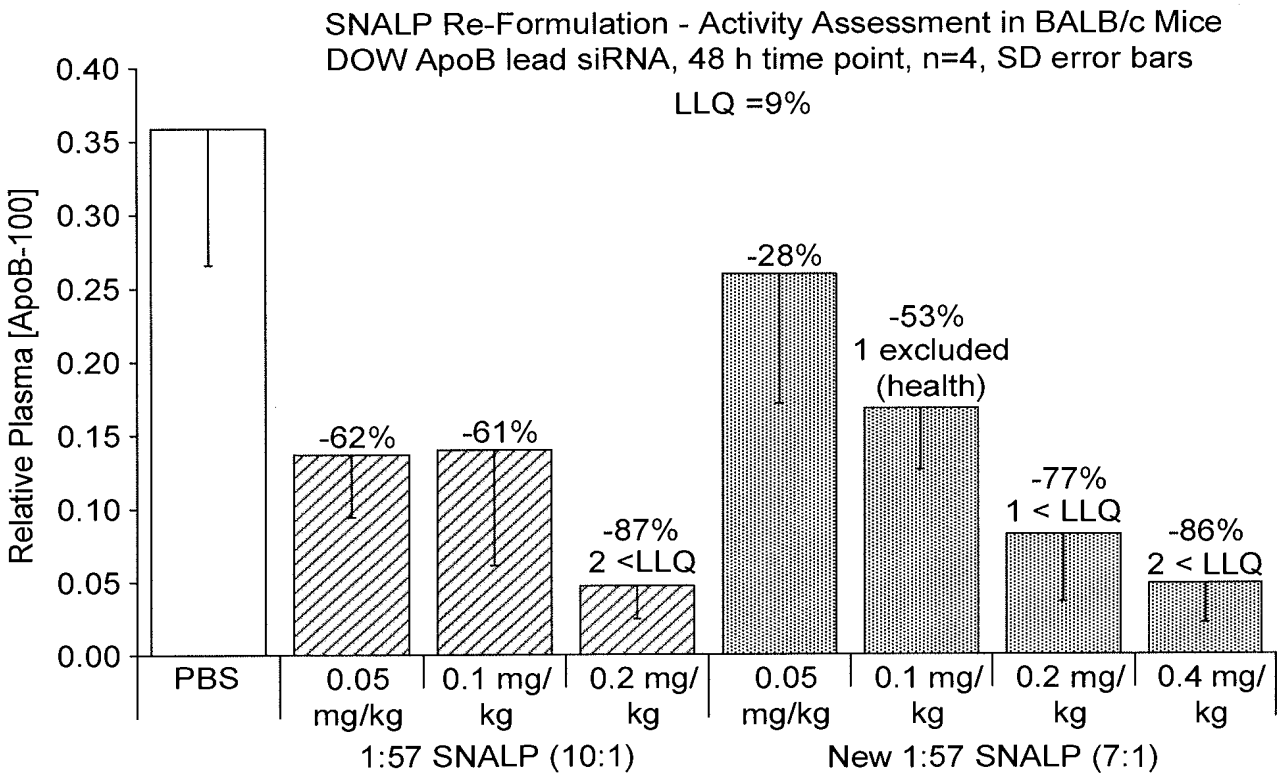


FIG. 11B



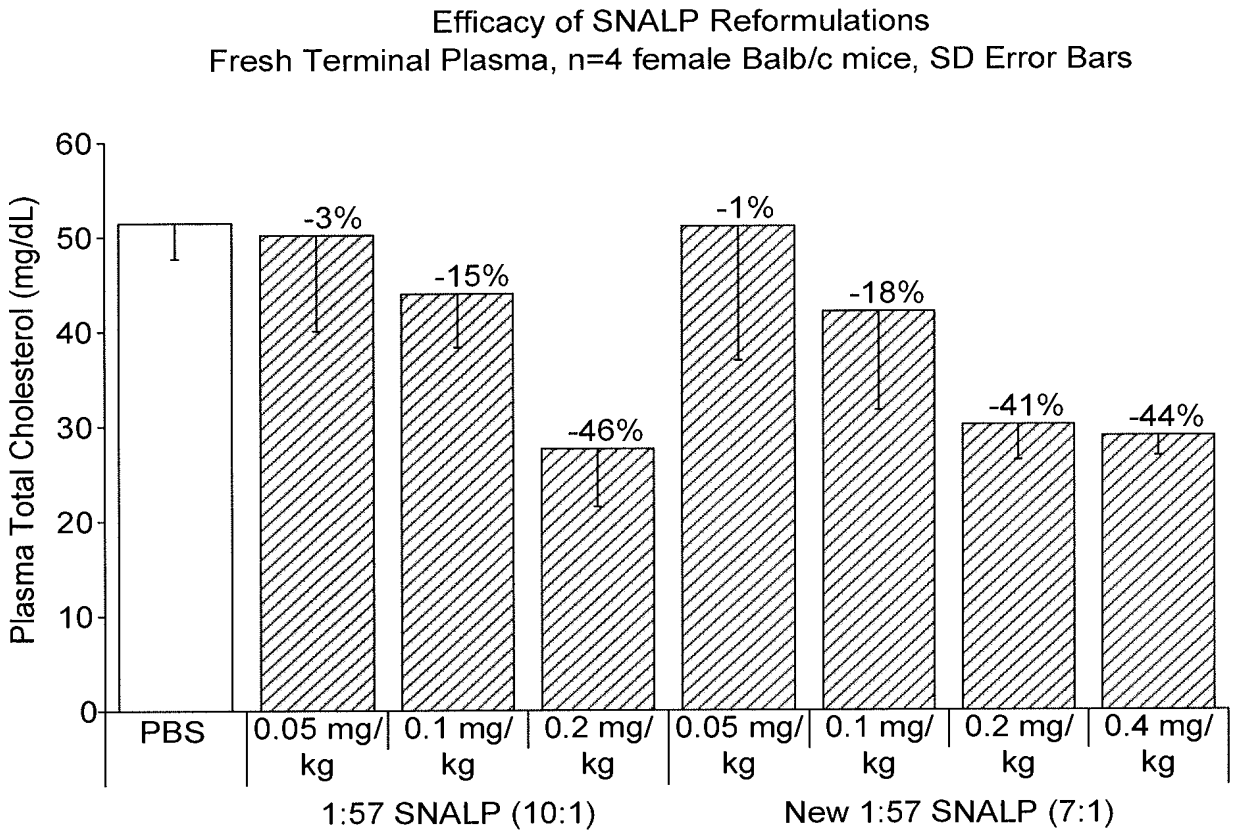

Efficacy of SNALP Reformulations
Fresh Terminal Plasma, n=4 female Balb/c mice, SD Error Bars
Plasma Total Cholesterol (mg/dL)
60
50
40
30
20
10
0
-3%
-15%
-46%
-1%
-18%
-41%
-44%
PBS
0.05 mg/ kg
0.1 mg/ kg
0.2 mg/ kg
0.05 mg/ kg
0.1 mg/ kg
0.2 mg/ kg
0.4 mg/ kg
1:57 SNALP (10:1)
New 1:57 SNALP (7:1)

FIG. 12

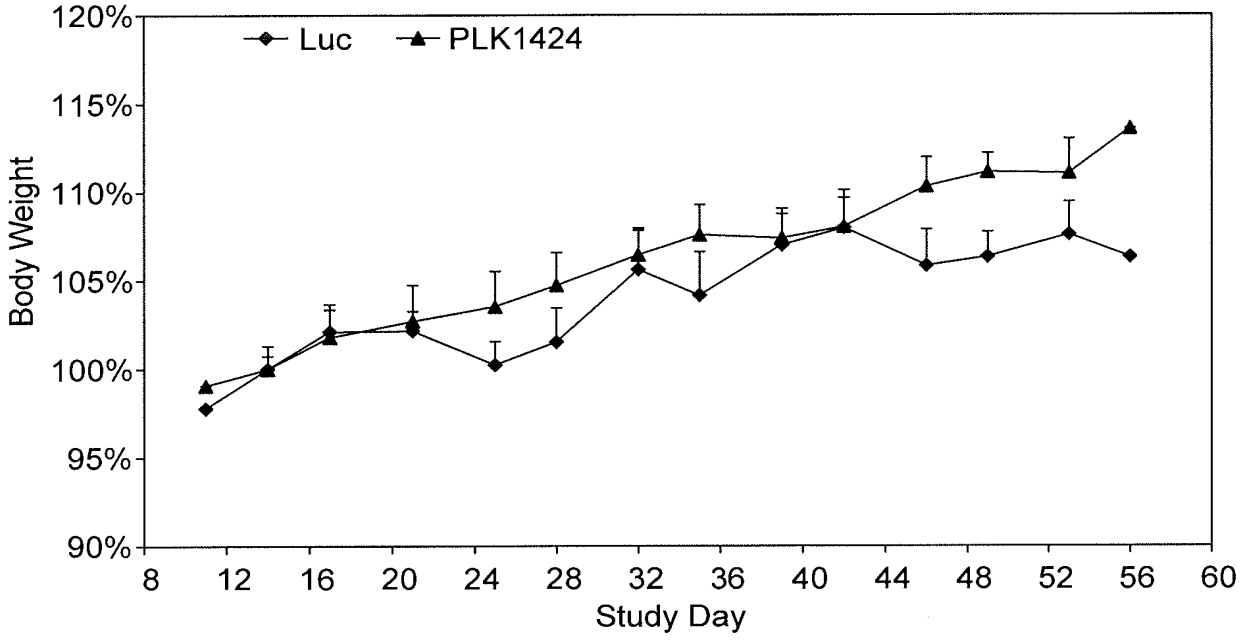

120%
115%
110%
105%
100%
95%
90%
Body Weight
Luc
PLK1424
8
12
16
20
24
28
32
36
40
44
48
52
56
60
Study Day

FIG. 13

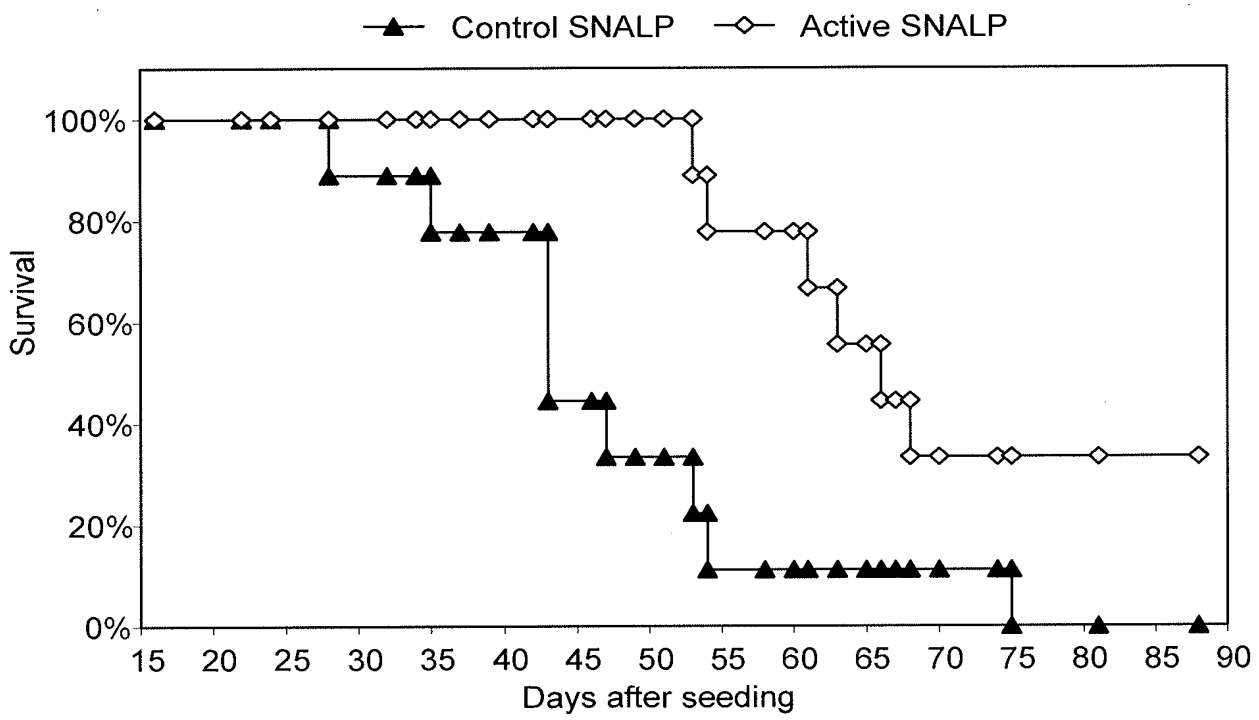

Control SNALP
Active SNALP
100%
80%
60%
40%
20%
0%
Survival
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
Days after seeding

FIG. 14

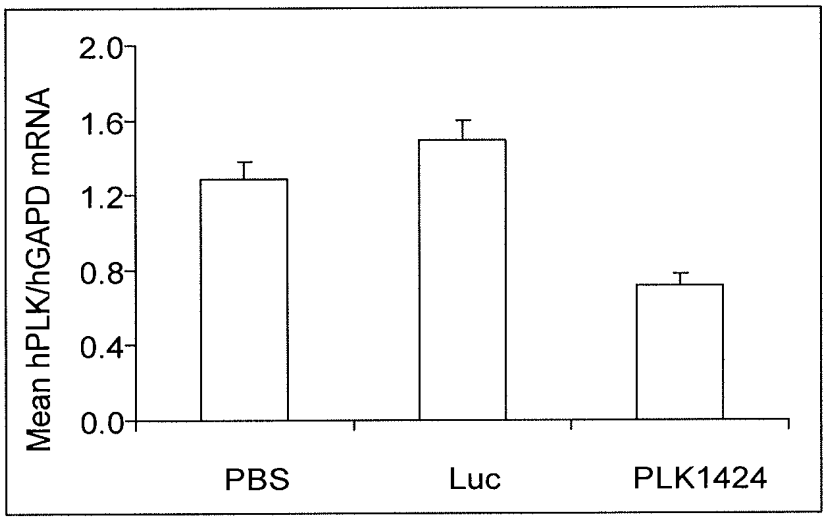


FIG. 15

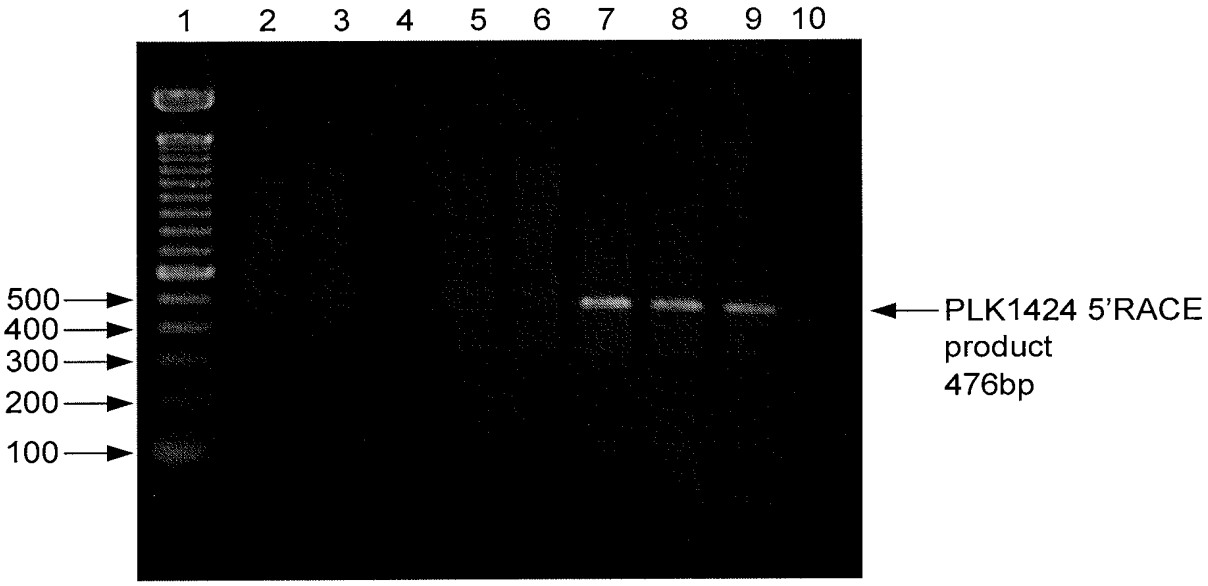
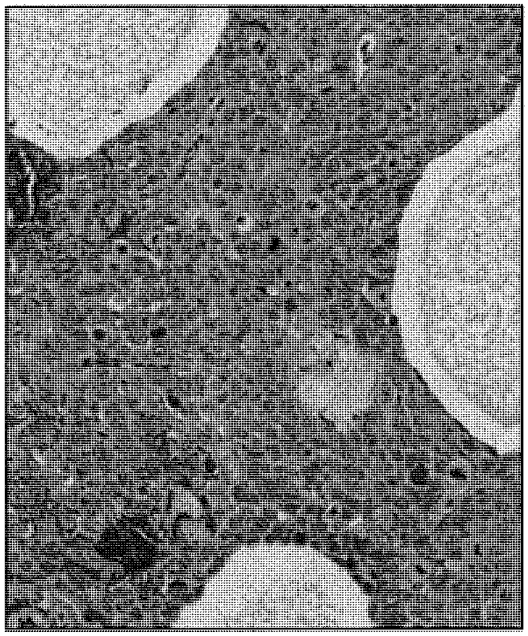
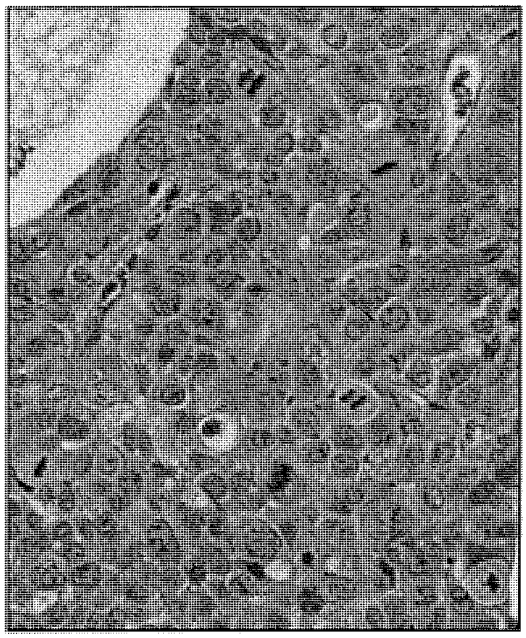

1 2 3 4 5 6 7 8 9 10
500
400
300
200
100
PLK1424 5'RACE
product
476bp

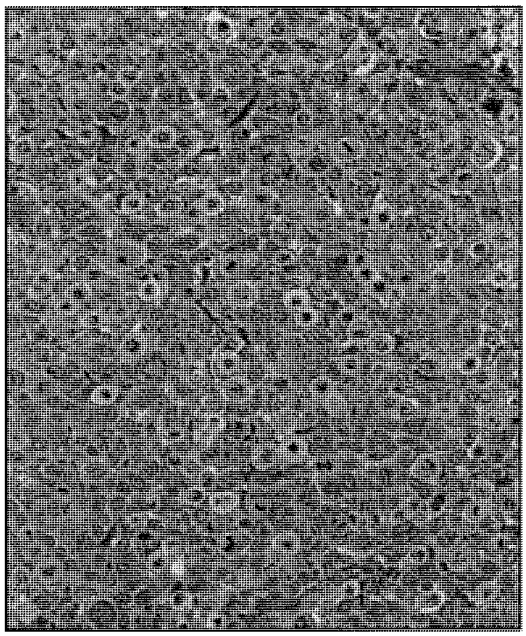
FIG. 16



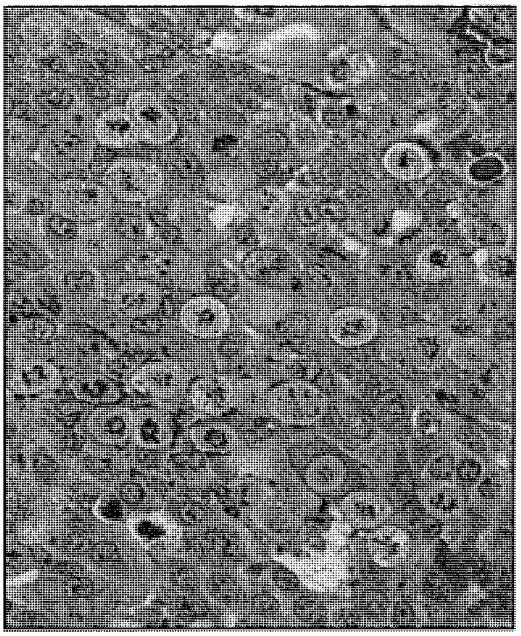
x200 mag



x400 mag



x200 mag



x400 mag

FIG. 17

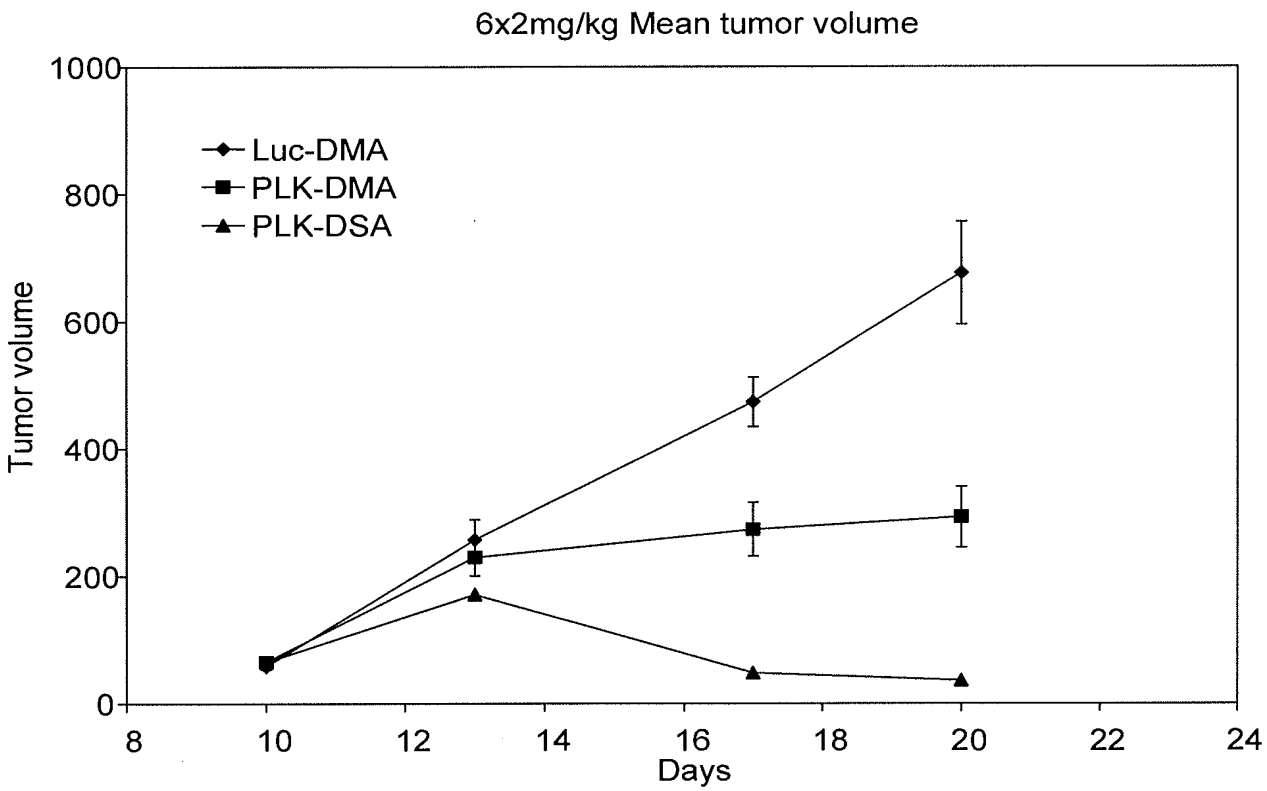

6x2mg/kg Mean tumor volume
Luc-DMA
PLK-DMA
PLK-DSA
Tumor volume
1000
800
600
400
200
0
8
10
12
14
16
18
20
22
24
Days

FIG. 18

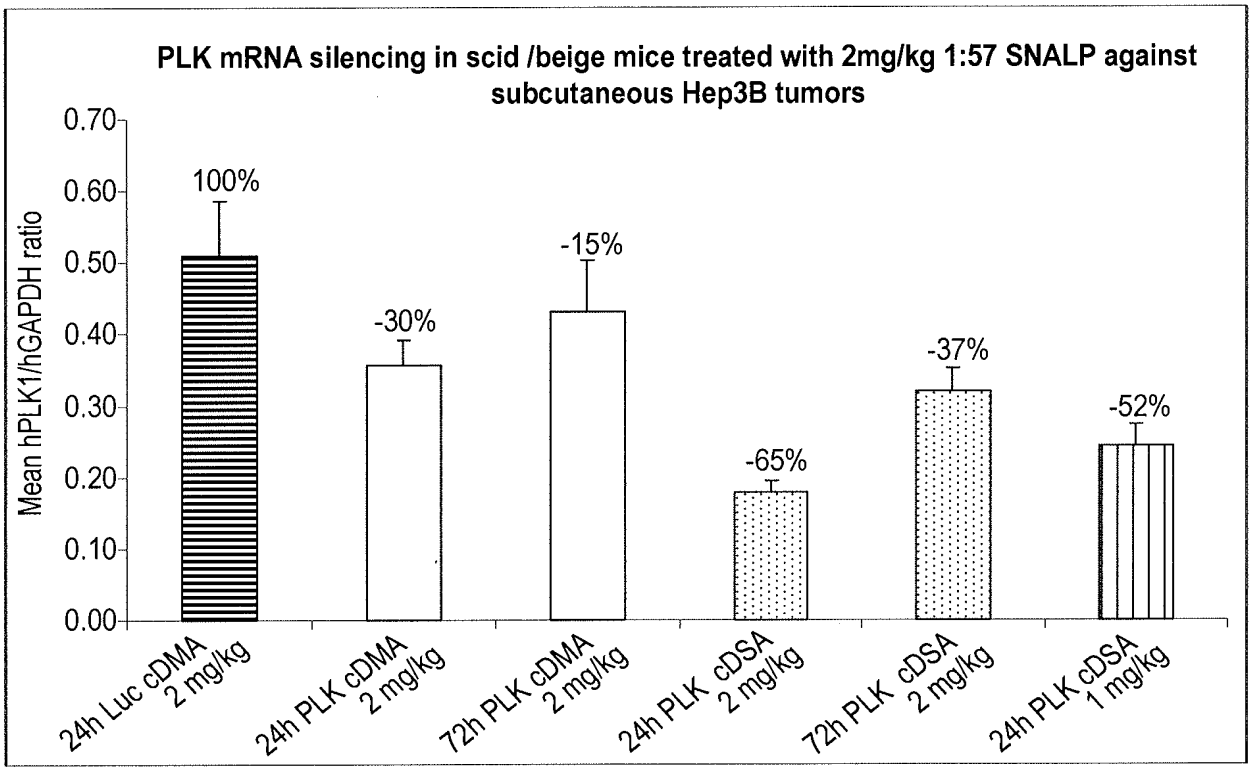

PLK mRNA silencing in scid /beige mice treated with 2mg/kg 1:57 SNALP against subcutaneous Hep3B tumors
Mean hPLK1/hGAPDH ratio
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
100%
-30%
-15%
-65%
-37%
-52%
24h Luc cDMA 2 mg/kg
24h PLK cDMA 2 mg/kg
72h PLK cDMA 2 mg/kg
24h PLK cDSA 2 mg/kg
72h PLK cDSA 2 mg/kg
24h PLK cDSA 1 mg/kg

FIG. 19

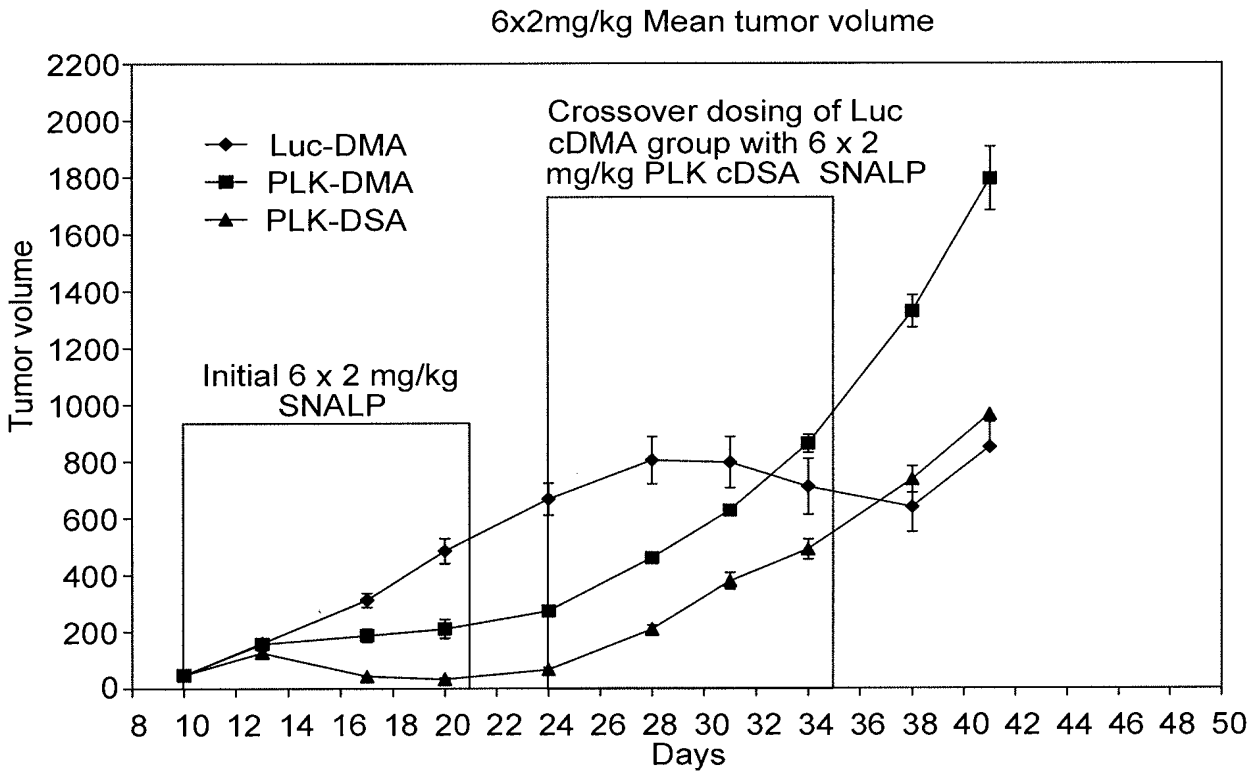

6x2mg/kg Mean tumor volume
Tumor volume
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Luc-DMA
PLK-DMA
PLK-DSA
Initial 6 x 2 mg/kg SNALP
Crossover dosing of Luc cDMA group with 6 x 2 mg/kg PLK cDSA SNALP
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Days

FIG. 20

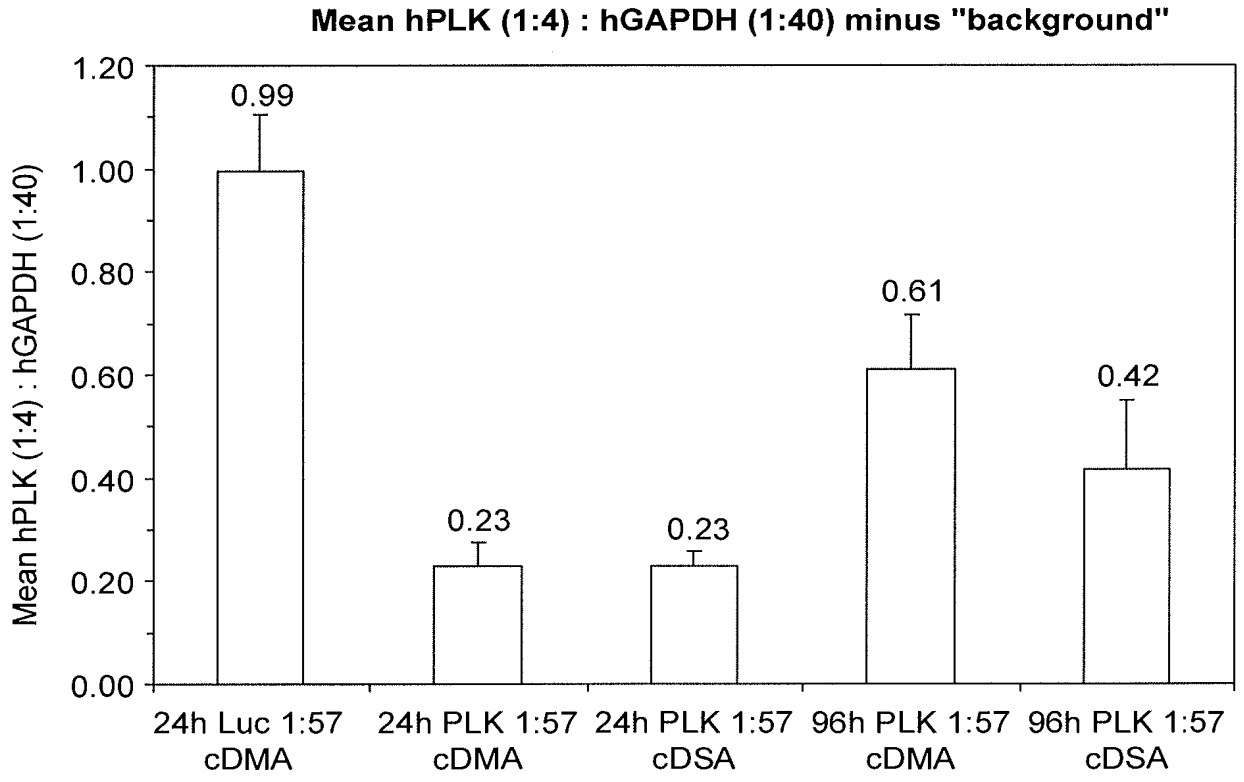

Mean hPLK (1:4) : hGAPDH (1:40) minus "background"
Mean hPLK (1:4) : hGAPDH (1:40)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.99
0.23
0.23
0.61
0.42
24h Luc 1:57 cDMA
24h PLK 1:57 cDMA
24h PLK 1:57 cDSA
96h PLK 1:57 cDMA
96h PLK 1:57 cDSA

FIG. 21

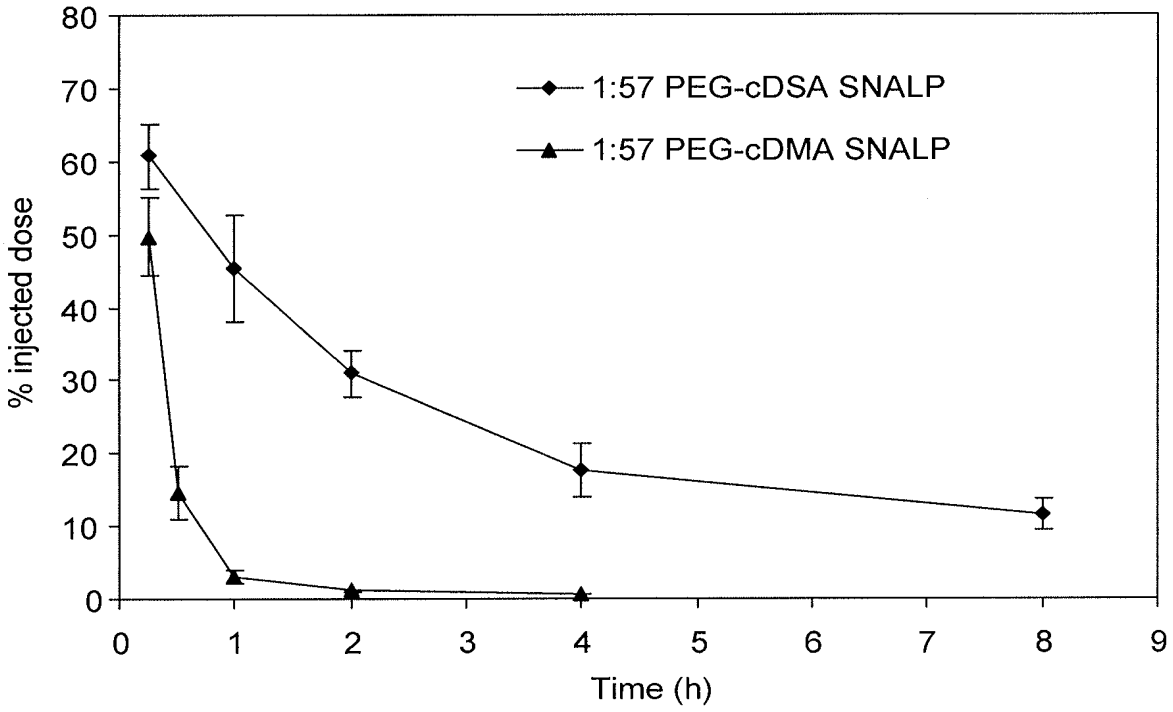

80
70
60
50
40
30
20
10
0
% injected dose
0
1
2
3
4
5
6
7
8
9
Time (h)
1:57 PEG-cDSA SNALP
1:57 PEG-cDMA SNALP

FIG. 22

Electronic Acknowledgement Receipt

EFS ID:	19896438
Application Number:	14462441
International Application Number:	
Confirmation Number:	6562
Title of Invention:	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
First Named Inventor/Applicant Name:	Edward Yaworski
Customer Number:	20350
Filer:	Joe Chao-Peng Hao/Judith Cotham
Filer Authorized By:	Joe Chao-Peng Hao
Attorney Docket Number:	86399-007740US-913296
Receipt Date:	18-AUG-2014
Filing Date:	
Time Stamp:	20:03:24
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		86399_007740_913296_trans_ADS_POA_DECS_081814.pdf	1150699 5f0543c060043814bfed8b78a028fed140db1af1	yes	15

	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Transmittal of New Application		1	1	
	Oath or Declaration filed		2	6	
	Application Data Sheet		7	13	
	Power of Attorney		14	14	
	Assignee showing of ownership per 37 CFR 3.73.		15	15	
Warnings:					
Information:					
2		86399_007740_913296_spec_081814.pdf	7229838 5f933d8328103583db1273a5bbb5cde1ee78ac2e	yes	119
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Specification		1	113	
	Claims		114	118	
	Abstract		119	119	
Warnings:					
Information:					
3	Drawings-only black and white line drawings	86399_007740_913296_figs_081814.pdf	1326181 eb92b5d84728e6985f574320c8e194fb82c310f0	no	24
Warnings:					
Information:					
4	Sequence Listing (Text File)	86399_007730_879491_seq_110613.TXT	4241	no	0
Warnings:					
Information:					
		Total Files Size (in bytes):	9710959		

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.

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============================================================
Sequence Listing was accepted.
If you need help call the Patent Electronic Business Center at (866)
217-9197 (toll free).
Reviewer: Anjum, Durreshwar (CGI Federal)
Timestamp: [year=2014; month=8; day=19; hr=11; min=23; sec=40; ms=858; ]
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Validated By CRFValidator v 1.0.4

Application No: 14462441 Version No: 1.0

Input Set:

Output Set:

Started: 2014-08-18 20:03:30.806
Finished: 2014-08-18 20:03:32.893
Elapsed: 0 hr(s) 0 min(s) 2 sec(s) 87 ms
Total Warnings: 39
Total Errors: 0
No. of SeqIDs Defined: 7
Actual SeqID Count: 7

Error code		Error Description
W	213	Artificial or Unknown found in <213> in SEQ ID (1)
W	447	n or Xaa used, for: SEQID(1) on line number 51
W	447	n or Xaa used, for: SEQID(1) on line number 51
W	447	n or Xaa used, for: SEQID(1) on line number 51
W	447	n or Xaa used, for: SEQID(1) on line number 51
W	447	n or Xaa used, for: SEQID(1) on line number 51
W	213	Artificial or Unknown found in <213> in SEQ ID (2)
W	447	n or Xaa used, for: SEQID(2) on line number 76
W	447	n or Xaa used, for: SEQID(2) on line number 76
W	447	n or Xaa used, for: SEQID(2) on line number 76
W	447	n or Xaa used, for: SEQID(2) on line number 76
W	447	n or Xaa used, for: SEQID(2) on line number 76
W	213	Artificial or Unknown found in <213> in SEQ ID (3)
W	447	n or Xaa used, for: SEQID(3) on line number 96
W	447	n or Xaa used, for: SEQID(3) on line number 96
W	447	n or Xaa used, for: SEQID(3) on line number 96
W	213	Artificial or Unknown found in <213> in SEQ ID (4)
W	447	n or Xaa used, for: SEQID(4) on line number 120
W	447	n or Xaa used, for: SEQID(4) on line number 120
W	447	n or Xaa used, for: SEQID(4) on line number 120

Input Set:

Output Set:

Started: 2014-08-18 20:03:30.806
Finished: 2014-08-18 20:03:32.893
Elapsed: 0 hr(s) 0 min(s) 2 sec(s) 87 ms
Total Warnings: 39
Total Errors: 0
No. of SeqIDs Defined: 7
Actual SeqID Count: 7

Error code	Error Description
W 447	n or Xaa used, for: SEQID(4) on line number 120
W 213	Artificial or Unknown found in <213> in SEQ ID (5)
W 447	n or Xaa used, for: SEQID(5) on line number 147
W 447	n or Xaa used, for: SEQID(5) on line number 147
W 447	n or Xaa used, for: SEQID(5) on line number 147 This error has occured more than 20 times, will not be displayed
W 213	Artificial or Unknown found in <213> in SEQ ID (6)
W 213	Artificial or Unknown found in <213> in SEQ ID (7)

SEQUENCE LISTING

<110> MacLachlan, Ian
Yaworski, Edward
Lam, Kieu

<120> Novel Lipid Formulations for Nucleic
Acid Delivery

<130> 86399-879491
<140> US 14/462,441
<141> 2014-08-18

<150> US 13/928,309
<151> 2013-06-26

<150> US 61/045,228
<151> 2008-04-15

<150> US 12/424,367
<151> 2009-04-15

<150> US 13/253,917
<151> 2011-10-05

<160> 7

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<223> synthetic E5 kinesin-related protein 2263 U/U
siRNA sense strand of siRNA duplex

<220>
<223> Description of Combined DNA/RNA Molecule:synthetic E5
kinesin-related protein 2263 U/U siRNA sense strand of
siRNA duplex

<221> modified_base
<222> (2)...(19)
<223> n = um

<221> modified_base
<222> (20)...(21)
<223> n = deoxythimidine (dT)

<400> 1
cngaagaccn gaagacaann n 21

<210> 2
<211> 21
<212> DNA

<213> Artificial Sequence

<220>
<223> synthetic E5 kinesin-related protein 2263 U/U
siRNA antisense strand of siRNA duplex

<220>
<223> Description of Combined DNA/RNA Molecule:synthetic E5
kinesin-related protein 2263 U/U siRNA antisense strand
of siRNA duplex

<221> modified_base
<222> (8)...(16)
<223> n = um

<221> modified_base
<222> (20)...(21)
<223> n = deoxythimidine (dT)

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auugucunca ggncuncagn n 21

<210> 3
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic apolipoprotein B (ApoB) 10048 U2/2 G1/2
siRNA sense strand of siRNA duplex

<221> modified_base
<222> (4)...(4)
<223> n = gm

<221> modified_base
<222> (8)...(14)
<223> n = um

<400> 3
agunucanca cacngaauac c 21

<210> 4
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic apolipoprotein B (ApoB) 10048 U2/2 G1/2
siRNA antisense strand of siRNA duplex

<221> modified_base
<222> (4)...(4)
<223> n = um

<221> modified_base
<222> (7)...(9)

<223> n = gm

<221> modified_base
<222> (19)...(19)
<223> n = um

<400> 4
uauncanunu gaugacacnu g 21

<210> 5
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic polo-like kinase 1 (PLK-1) PLK1424 U4/GU
and PLK1424 U4/G siRNA sense strand of siRNA
duplex

<220>
<223> Description of Combined DNA/RNA Molecule:synthetic
polo-like kinase 1 (PLK-1) PLK1424 U4/GU and PLK1424
U4/G siRNA sense strand of siRNA duplex

<221> modified_base
<222> (4)...(18)
<223> n = um

<221> modified_base
<222> (20)...(21)
<223> n = deoxythimidine (dT), u or a ribonucleotide
complementary to target complementary sequence

<400> 5
agancacccn ccunaaanan n 21

<210> 6
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic polo-like kinase 1 (PLK-1) PLK1424 U4/GU
siRNA antisense strand of siRNA duplex

<220>
<223> Description of Combined DNA/RNA Molecule:synthetic
polo-like kinase 1 (PLK-1) PLK1424 U4/GU siRNA
antisense strand of siRNA duplex

<221> modified_base
<222> (8)...(8)
<223> n = gm

<221> modified_base
<222> (17)...(17)
<223> n = um

<221> modified_base
<222> (20)...(21)
<223> n = deoxythimidine (dT), u or a ribonucleotide
complementary to target sequence

<400> 6
uauuuaanga gggugancun n 21

<210> 7
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic polo-like kinase 1 (PLK-1) PLK1424 U4/G
siRNA antisense strand of siRNA duplex

<220>
<223> Description of Combined DNA/RNA Molecule:synthetic
polo-like kinase 1 (PLK-1) PLK1424 U4/G siRNA
antisense strand of siRNA duplex

<221> modified_base
<222> (9)...(15)
<223> n = gm

<221> modified_base
<222> (20)...(21)
<223> n = deoxythimidine (dT), u or a ribonucleotide
complementary to target sequence

<400> 7
uauuuaagna gngunaucun n 21

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 14/462,441

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	280
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	600
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	720
TOTAL CLAIMS (37 CFR 1.16(i))	58 minus 20 =	* 38			OR	x 80 =	3040
INDEPENDENT CLAIMS (37 CFR 1.16(h))	3 minus 3 =	*				x 420 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						400
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							780
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	5820

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 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 NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
14/462,441	08/18/2014	1674	0.00	86399-007740US-913296	46	3

CONFIRMATION NO. 6562

20350
KILPATRICK TOWNSEND & STOCKTON LLP
TWO EMBARCADERO CENTER
EIGHTH FLOOR
SAN FRANCISCO, CA 94111-3834

FILING RECEIPT

Date Mailed: 08/29/2014

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Edward Yaworski, Maple Ridge, CANADA;
Kieu Lam, Surrey, CANADA;
Lloyd Jeffs, Delta, CANADA;
Lorne Palmer, Vancouver, CANADA;
Ian MacLachlan, Mission, CANADA;

Applicant(s)

Protiva Biotherapeutics, Inc., Burnaby, CANADA

Power of Attorney: The patent practitioners associated with Customer Number 20350

Domestic Priority data as claimed by applicant

This application is a CON of 13/928,309 06/26/2013 PAT 8822668
which is a CON of 13/253,917 10/05/2011 PAT 8492359
which is a CON of 12/424,367 04/15/2009 PAT 8058069
which claims benefit of 61/045,228 04/15/2008

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access - A proper **Authorization to Permit Access to Application by Participating Offices** (PTO/SB/39 or its equivalent) has been received by the USPTO.

If Required, Foreign Filing License Granted: 08/20/2014

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/462,441**

Projected Publication Date: To Be Determined - pending completion of Missing Parts

Non-Publication Request: No

Early Publication Request: No

Title

NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

Preliminary Class

514

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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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
14/462,441	08/18/2014	Edward Yaworski	86399-007740US-913296

CONFIRMATION NO. 6562

20350
KILPATRICK TOWNSEND & STOCKTON LLP
TWO EMBARCADERO CENTER
EIGHTH FLOOR
SAN FRANCISCO, CA 94111-3834

FORMALITIES LETTER

OC000000070312704

Date Mailed: 08/29/2014

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The statutory basic filing fee is missing.
- The application search fee must be submitted.
- The application examination fee must be submitted.
- Additional claim fees of $ **3820** as an undiscounted entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.
- Surcharge as set forth in 37 CFR 1.16(f) must be submitted.
  The surcharge is due for any one of:
  - late submission of the basic filing fee, search fee, or examination fee,
  - late submission of inventor's oath or declaration,
  - filing an application that does not contain at least one claim on filing, or
  - submission of an application filed by reference to a previously filed application.

SUMMARY OF FEES DUE:

The fee(s) required within **TWO MONTHS** from the date of this Notice to avoid abandonment is/are itemized below. No entity status discount is in effect. If applicant is qualified for small entity status, a written assertion of small entity status must be submitted to establish small entity status. (See 37 CFR 1.27). If applicant is qualified for micro entity status, an acceptable Certification of Micro Entity Status must be submitted to establish micro entity status. (See 37 CFR 1.29 and forms PTO/SB/15A and 15B.)

- $ **280** basic filing fee.
- $ **400** for **5** electronically equivalent pages in excess of **100** application size fee.
- $ **140** surcharge.
- $ **600** search fee.
- $ **720** examination fee.
- $ **3040** for **38** total claims over 20.

- $ **780** for multiple dependent claim surcharge.
- $(**0**) previous unapplied payment amount.
- $ **5960** TOTAL FEE BALANCE DUE.

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

Replies should be mailed to:

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web, including a copy of this Notice and selecting the document description "Applicant response to Pre-Exam Formalities Notice". https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

/nfissha/

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

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
14462441	

Applicant(s) Edward Yaworski

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT	
	Indep	Depend	Indep	Depend	Indep	Depend
1	1					
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Total Indep	3		0		0	
Total Depend	55	↵	0	↵	0	↵
Total Claims	58		0		0	

CLAIMS	*		*		*	
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I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on February 26, 2015.

KILPATRICK TOWNSEND & STOCKTON LLP

By: /Judith Cotham/
Judith Cotham

PATENT
Attorney Docket No.: 86399-007740US-913296

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Yaworski *et al.*

Application No.: 14/462,441

Filed: August 18, 2014

For: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

Customer No.: 20350

Confirmation No. 6562

Examiner: Not yet assigned

Art Unit: Not yet assigned

PRELIMINARY AMENDMENT

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

Commissioner:

Prior to examination of the above-referenced application, please enter the following amendments and remarks.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks begin on page 5 of this paper.

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1-46. (Canceled)

47. (New) A nucleic acid-lipid particle comprising:

(a) a nucleic acid;

(b) a cationic lipid comprising from 50 mol % to 85 mol % of the total lipid present in the particle;

(c) a non-cationic lipid comprising from 13 mol % to 49.5 mol % of the total lipid present in the particle; and

(d) a conjugated lipid that inhibits aggregation of particles comprising from 0.5 mol % to 2 mol % of the total lipid present in the particle.

48. (New) The nucleic acid-lipid particle of claim 47, wherein the nucleic acid comprises an interfering RNA, mRNA, an antisense oligonucleotide, a ribozyme, a plasmid, an immunostimulatory oligonucleotide, or mixtures thereof.

49. (New) The nucleic acid-lipid particle of claim 48, wherein the interfering RNA comprises a small interfering RNA (siRNA), an asymmetrical interfering RNA (aiRNA), a microRNA (miRNA), or mixtures thereof.

50. (New) The nucleic acid-lipid particle of claim 47, wherein the cationic lipid comprises from 50 mol % to 65 mol % of the total lipid present in the particle.

51. (New) The nucleic acid-lipid particle of claim 47, wherein the non-cationic lipid comprises a mixture of a phospholipid and cholesterol or a derivative thereof.

52. (New) The nucleic acid-lipid particle of claim 51, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

53. (New) The nucleic acid-lipid particle of claim 51, wherein the phospholipid comprises from 3 mol % to 15 mol % of the total lipid present in the particle.

54. (New) The nucleic acid-lipid particle of claim 51, wherein the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the particle.

55. (New) The nucleic acid-lipid particle of claim 47, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

56. (New) The nucleic acid-lipid particle of claim 55, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

57. (New) The nucleic acid-lipid particle of claim 56, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

58. (New) The nucleic acid-lipid particle of claim 47, wherein the conjugated lipid that inhibits aggregation of particles comprises from 1 mol % to 2 mol % of the total lipid present in the particle.

59. (New) The nucleic acid-lipid particle of claim 47, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

60. (New) A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 47 and a pharmaceutically acceptable carrier.

61. (New) A method for introducing a nucleic acid into a cell, the method comprising:

contacting the cell with a nucleic acid-lipid particle of claim 47.

62. (New) A method for the *in vivo* delivery of a nucleic acid, the method comprising:

administering to a mammalian subject a nucleic acid-lipid particle of claim 47.

63. (New) A method for treating a disease or disorder in a mammalian subject in need thereof, the method comprising:

administering to the mammalian subject a therapeutically effective amount of a nucleic acid-lipid particle of claim 47.

64. (New) The method of claim 63, wherein the disease or disorder is a viral infection.

65. (New) The method of claim 63, wherein the disease or disorder is a liver disease or disorder.

66. (New) The method of claim 63, wherein the disease or disorder is cancer.

REMARKS

After entry of this amendment, claims 47-66 are pending in this application and are presented for examination. Claims 1-46 have been canceled without prejudice to future prosecution. Claims 47-66 are newly added.

New claim 47 finds support, for example, in original claim 1. New claims 48 and 49 find support, for example, on page 13, lines 25-32 and on page 36, lines 22-24 of the instant specification. New claim 50 finds support, for example, on page 25, lines 7-11 of the instant specification. New claim 51 finds support, for example, in original claim 13. New claim 52 finds support, for example, in original claim 14. New claim 53 finds support, for example, on page 28, lines 9-10 of the instant specification. New claim 54 finds support, for example, on page 69, lines 3-4 of the instant specification. New claim 55 finds support, for example, in original claim 17. New claim 56 finds support, for example, in original claim 18. New claim 57 finds support, for example, in original claim 19. New claim 58 finds support, for example, in original claim 21. New claim 59 finds support, for example, in original claim 23. New claim 60 finds support, for example, in original claim 26. New claim 61 finds support, for example, in original claim 41. New claim 62 finds support, for example, in original claim 43. New claim 63 finds support, for example, in original claim 45. New claims 64-66 find support, for example, in original claim 46. As such, no new matter has been introduced with the foregoing amendments.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 925-472-5000.

Respectfully submitted,

/Joe C. Hao/

Joe C. Hao
Reg. No. 55,246

KILPATRICK TOWNSEND & STOCKTON LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111-3834
Tel: 925-472-5000
Fax: 415-576-0300
KILPATRICK TOWNSEND 66942357 1

PTO/SB/22 (03-13)
Approved for use through 3/31/2013. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)

Docket Number (Optional): 86399-007740US-913296

Application Number: 14/462,441

Filed: August 18, 2014

For: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

Art Unit: 1674

Examiner:

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above-identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

	Fee	Small Entity Fee	Micro Entity Fee	
☐ One month (37 CFR 1.17(a)(1))	$200	$100	$50	$
☐ Two months (37 CFR 1.17(a)(2))	$600	$300	$150	$
☐ Three months (37 CFR 1.17(a)(3))	$1,400	$700	$350	$
☑ Four months (37 CFR 1.17(a)(4))	$2,200	$1,100	$550	$ 2200
☐ Five months (37 CFR 1.17(a)(5))	$3,000	$1,500	$750	$

☐ 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.

☐ A check in the amount of the fee is enclosed.

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

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☐ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number _____.

☑ Payment made via EFS-Web.

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.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71. 37 CFR 3.73(b) statement is enclosed (Form PTO/SB/96).

☑ attorney or agent of record. Registration number 55246.

☐ attorney or agent acting under 37 CFR 1.34. Registration number _____.

/Joe C. Hao/ — Signature

February 26, 2015 — Date

Joe C. Hao — Typed or printed name

925 472 5000 — Telephone Number

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.

☐ * Total of _____ forms are submitted.

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.

Electronic Patent Application Fee Transmittal

Application Number:	14462441
Filing Date:	18-Aug-2014
Title of Invention:	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
First Named Inventor/Applicant Name:	Edward Yaworski
Filer:	Joe Chao-Peng Hao/Judith Cotham
Attorney Docket Number:	86399-007740US-913296

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Utility application filing	1011	1	280	280
Utility Search Fee	1111	1	600	600
Utility Examination Fee	1311	1	720	720
Pages:				
Utility Appl Size fee per 50 sheets >100	1081	1	400	400
Claims:				
Miscellaneous-Filing:				
Late Filing Fee for Oath or Declaration	1051	1	140	140

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 4 months with $0 paid	1254	1	2200	2200
Miscellaneous:				
		Total in USD ($)		**4340**

Electronic Acknowledgement Receipt

EFS ID:	21614179
Application Number:	14462441
International Application Number:	
Confirmation Number:	6562
Title of Invention:	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
First Named Inventor/Applicant Name:	Edward Yaworski
Customer Number:	20350
Filer:	Joe Chao-Peng Hao/Judith Cotham
Filer Authorized By:	Joe Chao-Peng Hao
Attorney Docket Number:	86399-007740US-913296
Receipt Date:	26-FEB-2015
Filing Date:	18-AUG-2014
Time Stamp:	17:42:35
Application Type:	Utility under 35 USC 111(a)

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Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$4340
RAM confirmation Number	4303
Deposit Account	201430
Authorized User	HAO, JOE C.

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		86399_007740_913296_PRELIM_AMD_022615.pdf	185971 8bd08780e2ab9322afec98d745f44be92e8b172d	yes	5

Multipart Description/PDF files in .zip description

Document Description	Start	End
Preliminary Amendment	1	1
Claims	2	4
Applicant Arguments/Remarks Made in an Amendment	5	5

Warnings:

Information:

2	Extension of Time	86399_007740_913296_EOT_022615.pdf	1048968 1d31beae3479e8cb9185d13f52b8fa450ecef1db	no	2

Warnings:

Information:

3	Fee Worksheet (SB06)	fee-info.pdf	40401 1b3c105f68d8d73c95814afc46b221c340baf654	no	2

Warnings:

Information:

Total Files Size (in bytes):	1275340

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.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/462,441	Filing Date 08/18/2014	☐ To be Mailed

ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT **02/26/2015**

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 20	Minus	** 20	= 0	X $80 =	0
Independent (37 CFR 1.16(h))	* 1	Minus	***3	= 0	X $420 =	0
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	0

AMENDMENT

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
/TAMMY d. MCBETH BROWN/

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 14/462,441

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	280
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	600
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	720
TOTAL CLAIMS (37 CFR 1.16(i))	20 minus 20 =	*			OR	x 80 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 420 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						400
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	2000

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
14/462,441	08/18/2014	1674	2140	86399-007740US-913296	20	1

CONFIRMATION NO. 6562

UPDATED FILING RECEIPT

20350
KILPATRICK TOWNSEND & STOCKTON LLP
TWO EMBARCADERO CENTER
EIGHTH FLOOR
SAN FRANCISCO, CA 94111-3834

OC00000073801488

Date Mailed: 03/09/2015

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Edward Yaworski, Maple Ridge, CANADA;
Kieu Lam, Surrey, CANADA;
Lloyd Jeffs, Delta, CANADA;
Lorne Palmer, Vancouver, CANADA;
Ian MacLachlan, Mission, CANADA;

Applicant(s)

Protiva Biotherapeutics, Inc., Burnaby, CANADA

Power of Attorney: The patent practitioners associated with Customer Number 20350

Domestic Priority data as claimed by applicant

This application is a CON of 13/928,309 06/26/2013 PAT 8822668
which is a CON of 13/253,917 10/05/2011 PAT 8492359
which is a CON of 12/424,367 04/15/2009 PAT 8058069
which claims benefit of 61/045,228 04/15/2008

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access - A proper **Authorization to Permit Access to Application by Participating Offices** (PTO/SB/39 or its equivalent) has been received by the USPTO.

If Required, Foreign Filing License Granted: 08/20/2014

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 14/462,441**

Projected Publication Date: 06/18/2015

Non-Publication Request: No

Early Publication Request: No

Title

NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

Preliminary Class

514

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

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Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
14/462,441	08/18/2014	Edward Yaworski	86399-007740US-913296

CONFIRMATION NO. 6562

20350
KILPATRICK TOWNSEND & STOCKTON LLP
TWO EMBARCADERO CENTER
EIGHTH FLOOR
SAN FRANCISCO, CA 94111-3834

PUBLICATION NOTICE

Title:NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

Publication No.US-2015-0164799-A1
Publication Date:06/18/2015

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 Office of Public Records. The Office of Public Records can be reached by telephone at (703) 308-9726 or (800) 972-6382, by facsimile at (703) 305-8759, by mail addressed to the United States Patent and Trademark Office, Office of Public Records, 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 http://pair.uspto.gov/. 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

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/462,441	08/18/2014	Edward Yaworski	86399-007740US-913296	6562

20350 7590 10/28/2015

KILPATRICK TOWNSEND & STOCKTON LLP
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EXAMINER
WHITEMAN, BRIAN A

ART UNIT	PAPER NUMBER
1674	

NOTIFICATION DATE	DELIVERY MODE
10/28/2015	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):

ipefiling@kilpatricktownsend.com
jlhice@kilpatrick.foundationip.com

Office Action Summary	Application No. 14/462,441	Applicant(s) YAWORSKI ET AL.	
	Examiner BRIAN WHITEMAN	Art Unit 1674	AIA (First Inventor to File) Status No

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

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ 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.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *47-66* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *47-66* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, 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.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *8/18/14* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ 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. _____.

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)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO internet Web site contains terminal disclaimer forms which may be used. Please visit http://www.uspto.gov/forms/. The filing date of the application will determine what form should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Claims 47-60 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-24 of U.S. Patent No. 9,006,417. Although the claims at issue are not identical, they are not patentably distinct from each other because both set of claims are directed to a composition comprising a nucleic acid-lipid particle comprising (a) a nucleic acid, (b) a cationic lipid, (c) a non-cationic lipid and (d) a conjugated lipid. The nucleic acid can be an siRNA. The percentage recited in b, c and d in both claims set embrace a similar range. In addition, both set of claims embrace using the same type of conjugated lipid, phospholipid and cholesterol or derivative thereof.

Claims 47-60 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-21 of U.S. Patent No. 8,492,359. Although the claims at issue are not identical, they are not patentably distinct from each other because both set of claims are directed to a composition comprising a nucleic acid-lipid particle comprising (a) a nucleic acid, (b) a cationic lipid, (c) a non-cationic lipid and (d) a

conjugated lipid. The nucleic acid can be an siRNA. The percentages listed in b, c and d in both claims set embrace a similar range. In addition, both set of claims embrace using the same type of conjugated lipid, phospholipid and cholesterol or derivative thereof.

Claims 47-66 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-23 of U.S. Patent No. 8,822,668. Although the claims at issue are not identical, they are not patentably distinct from each other because both set of claims are directed to a composition comprising a nucleic acid-lipid particle comprising (a) a nucleic acid, (b) a cationic lipid, (c) a non-cationic lipid and (d) a conjugated lipid. The nucleic acid can be an siRNA. The percentages listed in b, c and d in both claims set embrace a similar range. In addition, both set of claims embrace using the same type of conjugated lipid, phospholipid and cholesterol or derivative thereof. The composition can be used to treat a viral infection, liver disorder or cancer.

Claims 47-63 and 65 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-26 of U.S. Patent No. 8,236,943. Although the claims at issue are not identical, they are not patentably distinct from each other because both set of claims are directed to a composition comprising a nucleic acid-lipid particle comprising (a) a nucleic acid, (b) a cationic lipid, (c) a non-cationic lipid and (d) a conjugated lipid. The nucleic acid can be an siRNA. The percentages listed in b, c and d in both claims set embrace a similar range. In addition, both set of claims

embrace using the same type of conjugated lipid, phospholipid and cholesterol or derivative thereof. The composition can be used to treat a viral infection, liver disorder or cancer.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Brian Whiteman whose telephone number 571-272-0764. The examiner can normally be reached on Monday-Thursday from 6:30 to 4:00 (Eastern Standard Time). The examiner can also be reached on alternate Fridays. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor Mark Shibuya can be reached on 571 272-0806. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

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).

/Brian Whiteman/

Primary Examiner, Art Unit 1674

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	Examiner BRIAN WHITEMAN	Art Unit 1674	Page 1 of 1

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*	D	US-8,236,943	08-2012	Lee et al.	536/24.5
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	F	US-			
	G	US-			
	H	US-			
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	J	US-			
	K	US-			
	L	US-			
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	Examiner	Art Unit	
	BRIAN WHITEMAN	1674	

SEARCHED			
Class	Subclass	Date	Examiner

INTERFERENCE SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES (INCLUDING SEARCH STRATEGY)		
	DATE	EXMR
WEST, STN, Inventor	10/21/2015	BW

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	Application Number	14/462,441
	Filing Date	August 18, 2014
	First Named Inventor	Ed Yaworski
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	Application Number	14/462,441
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	Art Unit	1674
	Examiner Name	Whiteman, Brian A.
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EXAMINER SIGNATURE			
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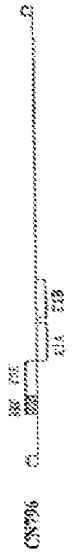
(54)【発明の名称】　細胞状態特異的反応エレメントを含むアデノウイルスベクター及びその使用方法

(57)【要約】
本発明は、アデノウイルス遺伝子上に細胞状態特異的転写制御を付与する細胞状態特異的転写制御エレメントを含むアデノウイルスベクターを提供する。「細胞状態」は一般に可逆的な生理学的及び／又は環境状態である。本発明は更に本ベクターを含む組成物及び宿主細胞、ならびに本ベクターを使用する方法も提供する。



【特許請求の範囲】

【請求項１】　細胞状態特異的ＴＲＥを含む転写制御エレメント（ＴＲＥ）の転写制御下にあるアデノウイルス遺伝子を含むアデノウイルスベクター。

【請求項２】　アデノウイルス遺伝子がウイルス複製に必須である請求項１のアデノウイルスベクター。

【請求項３】　アデノウイルス遺伝子が初期遺伝子である請求項２のアデノウイルスベクター。

【請求項４】　アデノウイルス遺伝子が後期遺伝子である請求項２のアデノウイルスベクター。

【請求項５】　アデノウイルス初期遺伝子がＥ１Ａである請求項３のアデノウイルスベクター。

【請求項６】　アデノウイルス初期遺伝子がＥ１Ｂである請求項３のアデノウイルスベクター。

【請求項７】　アデノウイルス初期遺伝子がＥ４である請求項３のアデノウイルスベクター。

【請求項８】　細胞状態特異的ＴＲＥがヒトのものである請求項１のアデノウイルスベクター。

【請求項９】　細胞状態特異的ＴＲＥが低酸素反応性エレメント（ＨＲＥ）を含む請求項１のアデノウイルスベクター。

【請求項１０】　ＨＲＥが配列番号１を含む、請求項９のアデノウイルスベクター。

【請求項１１】　細胞状態特異的ＴＲＥが細胞周期特異的エレメントを含む請求項１のアデノウイルスベクター。

【請求項１２】　細胞周期特異的エレメントがＥ２Ｆ－１遺伝子に由来する請求項１１のアデノウイルスベクター。

【請求項１３】　細胞状態特異的ＴＲＥが熱誘導エレメントを含む請求項１のアデノウイルスベクター。

【請求項１４】　更に細胞型特異的ＴＲＥを含む請求項１のアデノウイルスベクター。

【請求項１５】　細胞型特異的ＴＲＥが前立腺細胞特異的である請求項１４のアデノウイルスベクター。

【請求項１６】　前立腺細胞特異的ＴＲＥがＰＳＡ－ＴＲＥである請求項１５のアデノウイルスベクター。

【請求項１７】　更に第２細胞状態特異的ＴＲＥの転写制御下にあるトランス遺伝子を含む請求項１のアデノウイルスベクター。

【請求項１８】　細胞状態特異的ＴＲＥ及び細胞型特異的ＴＲＥを含むＴＲＥの転写制御下にあるアデノウイルス遺伝子を含むアデノウイルスベクター。

【請求項１９】　アデノウイルス遺伝子が初期遺伝子である請求項１８のアデノウイルスベクター。

【請求項２０】　初期遺伝子がＥ１Ａである請求項１９のアデノウイルスベクター。

【請求項２１】　細胞状態特異的ＴＲＥがＨＲＥを含み、細胞タイア特異的ＴＲＥがＰＳＡ－ＴＲＥである請求項２０のアデノウイルスベクター。

【請求項２２】　ＨＲＥが配列番号１を含み、そしてＰＳＡ－ＴＲＥが配列番号３の約５０３から約２０８６のヌクレオチド及び配列番号３の約５２８５から約５８３６までのヌクレオチドを含む、請求項２１のアデノウイルスベクター。

【請求項２３】　請求項１のアデノウイルスベクターを含む組成物。

【請求項２４】　更に医薬品として受け入れ可能な成分を含む請求項２３の組成物。

【請求項２５】　請求項１のアデノウイルスベクターを含む宿主細胞。

【請求項２６】　請求項１によるアデノウイルスと細胞を組合せ、それにより該アデノウイルスが増殖することを含む、細胞状態特異的ＴＲＥを機能できる細胞に特異的なアデノウイルスを増殖させる方法。

【請求項２７】　細胞状態特異的ＴＲＥを機能できる細胞を請求項１のアデノウイルスベクターと接触させ、それにより該ベクターを細胞内に侵入させることを含む、標的細胞に特異的な細胞傷害性を付与する方法。

【請求項２８】　請求項１のアデノウイルスベクターを細胞状態特異的ＴＲ

Eを機能できる腫瘍細胞内に導入し、そのなかでアデノウイルスベクターの導入が腫瘍増殖の抑制をもたらすことを含む腫瘍増殖を抑制する方法。

【発明の詳細な説明】

【０００１】

関連出願に関する相互参照

本出願は1998年９月10日出願の米国仮出願、特許出願番号60/099,791の優先権を主張する。従って、先出願はその全てが参照され、ここに取り込まれている。

【０００２】

政府後援研究により成された発明に関する権利状態

（適用外）

発明の分野

本発明はアデノウイルスベクターを用いる細胞トランスフェクションに関する、より具体的には、それは組織又は細胞の型に関係しない、細胞内でのアデノウイルスベクターの細胞状態特異的複製に関する。

【０００３】

従来の技術

多くの医学研究の進歩にも関わらず、癌はいまだに米国内に於ける第２の死因である。工業化国では、おおよそ５人に１人が癌によって死亡している。手術による切除、放射線治療や化学療法といった従来の臨床治療は失敗率が高く、特に固形癌での失敗率が高い。良性腫瘍を生ずる新生物は通常手術による病巣の除去によって完全に治癒できる。周辺組織の浸潤により明らかとなる様に、腫瘍が悪性化すると、根治は極めて困難になる。悪性腫瘍が転移した場合、根治することは殆どない。

【０００４】

基底細胞癌を除き、米国だけでも毎年百万人以上が新たに癌になり、米国内では毎年50万人以上が癌で死亡している。世界中で最も一般的な５つの癌は肺癌、胃癌、乳癌、結腸／直腸癌及び子宮頸癌であり、その毎年の新規患者合計数は６百万人以上である。

肺癌は、手術不能率が60％にも達すること、そして５年生存率が13％に過ぎないことなどから、最も難治性である固形癌の一つである。具体的には、全肺癌のほぼ半数が相当する腺癌は、大部分が化学－放射線耐性である。結腸直腸癌は３

番目に多い癌であり、２番目の癌死の原因である。膵臓癌は実際的には常に致死的である。即ち、これら癌にかかった多くの患者にとって現在の治療は満足できないものであり、そして予後は不良である。

【０００５】

肝細胞癌（ＨＣＣ又は悪性肝癌）は世界中で最も多く見られる癌の一つであり、特にアジアで問題である。肝細胞癌患者に関する治療法は明瞭ではない。治療法が改善され、肝臓移植が利用可能になっても、切除または移植による腫瘍除去によって治癒する患者は極一部である。大部分の患者にとっては、現行治療法は不満足であり、その予後も不良である。

【０００６】

乳癌は米国内に於ける最も一般的な癌の一つであり、毎年約182,000名が新たに発症し、ほぼ50,000名が死亡している。工業国では、約８人に１人の女性が乳癌になると予想されている。乳癌の死亡率は1930年来変わっていない。平均で毎年0.2%増加しているが、65歳以下の年齢の女性では平均0.3%/年減少している。Ｍａｒｃｈａｎｔ（１９９４）のＣｏｎｔｅｍｐｏｒａｒｙ Ｍａｎａｇｅｍｅｎｔ ｏｆ Ｂｒｅａｓｔ Ｄｉｓｅａｓｅ ＩＩ：Ｂｒｅａｓｔ Ｃａｎｃｅｒ、「Ｏｂｓｔｅｔｒｉｃｓ ａｎｄ Ｃｙｎｅｃｏｌｏｇｙ Ｃｌｉｎｉｃｓ ｏｆ Ｎｏｒｔｈ Ａｍｅｒｉｃａ」２１：５５５－５６０及びＣｏｌｄｉｔｚ（１９９３）の「Ｃａｎｃｅｒ Ｓｕｐｐｌ．」７１：１４８０－１４８９参照。

【０００７】

病気の理解は進んだにも関わらず、乳癌は医療介入に対し耐性を維持している。臨床上の最優先事項は早期診断であり、続いて通常型の介入、特に手術と化学療法である。この様な介入での成功は限定的であり、特に腫瘍が転移した患者では限定的である。より正確かつより効果的な、侵襲性の低い治療を提供に利用できる治療法の集積の改良が求められている。

【０００８】

前立腺癌は、1995年に米国内にて新たに約244,000名が診断され、そのうち約44,000が死亡すると考えられる、男性に於ける最も増加の著しい新生物である。

今日、男性では前立腺癌は最も多く診断される癌である。前立腺癌は潜在的である；多くの男性は病気の明瞭な徴候を示さない状態で前立腺癌を有している。これに伴い死亡率が高くなる。骨への癌転移（後期）は一般的であり、また殆ど致死的である。

【０００９】

現在の治療には、放射前立腺切除術、放射線治療、ホルモン枯渇及び化学療法が含まれている。残念なことに患者のおよそ80％では、前立腺癌の診断が確立した時点では既に骨転移が起こっており、従って外科的治療の効果は限定的である。ホルモン治療は失敗することが多く、時間と共にホルモン耐性癌細胞が生ずる。前立腺癌の治療には化学療法剤が利用されているが、その副作用を越える効果を持つものは無く、特に有効な薬剤併用治療も無いようである。一般に多くの前立腺癌は薬剤耐性で、ゆっくり増殖する性質を持っており、これが標準的な化学療法に対し非反応性にしている。

【００１０】

主要な、そして圧倒的な癌治療の障害は選択性の問題である；即ち正常細胞の機能には影響せずに腫瘍細胞の増殖を阻害する能力である。例えば、前立腺癌治療では、通常の化学療法の治療比率、又は殺滅された正常細胞に対する殺滅された腫瘍細胞の割合は1.5：１に過ぎない。この様なことから、新生物の治療と予防に適したより効果的な治療法及び医薬組成物が求められている。

【００１１】

固形癌は、腫瘍細胞が血管を形成する内皮細胞に比べ早く増殖することを一因とし、血管新生が不良な領域を含むことが多い。腫瘍細胞はこの様な低酸素条件下でも生存が可能であり、しばしば化学療法及び放射線治療に対し不応性である。子宮頸癌の最近の研究では、腫瘍の酸素状態は、患者年齢、閉経状態、臨床段階、大きさや組織学よりも重要な、単独の最重要予後因子であることを示している。Noeckelら、(1996) Semin. Radiat. Oncol. 6:1-8.

特に関心深いことは、より特異的な、狙いを定めた癌治療法であり、特に治療成功が難しい癌に適したものである。比較的非特異的であり、しばしば重い毒性をもたらす通常の癌治療に対し、特異的治療法は、健康な細胞をその無傷な状態

に残しながら悪性細胞を選択的に阻害又は殺滅しようとするものである。放射線耐性及び化学療法耐性腫瘍は特に難題であり、このタイプの腫瘍を治療する方法が求められている。

【００１２】

これら癌に関する一つの可能な方法は、所望する遺伝子を悪性細胞内に導入する遺伝子治療である。アデノウイルスベクターを含む様々なウイルスベクターが遺伝子治療の為の媒介体として開発されている。遺伝子治療を目的としたアデノウイルスベクター開発に於ける専らの関心事はアデノウイルスを所望する遺伝子を導入するための媒介体として利用することであり、それ自体をエフェクターとして利用することではない。アデノウイルスの複製は、主に宿主免疫反応の観点から望ましくない結果と見られている。複製欠損アデノウイルスによる癌治療では、宿主免疫反応は繰り返し投与期間を２つのレベルで制限している。第１の問題はアデノウイルス供与媒介体のカプシド蛋白質が免疫原性であることである。第２の問題は、ウイルスの後期遺伝子が形質導入された細胞においてしばしば発現し、それが細胞性免疫を惹起することである。即ち、繰り返し投与されるサイトカイン、腫瘍抑制遺伝子、リボザイム、自殺遺伝子、又はプロドラッグを活性薬物に変換する遺伝子の能力は、遺伝子運搬媒介体と運搬媒介体両方のウイルス遺伝子産物の持つ免疫原性、ならびに遺伝子発現の一過性の性質により制限されている。

【００１３】

癌治療ベクターとしてのアデノウイルスベクターの使用が報告されている。これら方法の幾つかは、アデノウイルスを選択的に複製させる（結果として細胞毒性を発揮させる）、組織（即ち細胞型）特異的転写制御エレメントを利用している。米国特許第5,698,443号；又WO95/11984号；WO96/17053号；WO96/34969号；WO98/35028号を参照。この方法は有用かつ有望であるが、組織特異的複製が不十分であるという別の治療上の問題が残っている。

【００１４】

癌性細胞以外にも、特定の望ましくない細胞又は組織を選択的に破壊することが望ましいことがしばしばある。しかし、侵襲的な手術以外に利用できる方法は

少なく、特にこの様な選択的細胞傷害性及び／又は抑制を可能にする非侵襲的方法は少ない。

ベクターに対する特異的な免疫学反応がその後の治療を妨げる前に、最少回数の投与により実質的に全癌性細胞を排除でき、そして特異的な、狙いを定めた癌根治治療での利用に好適なベクター構築体が求められている。更に、細胞のタイプ及び／又は解剖学的局在部位に関わらず、不要の細胞を選択的に破壊し、又は傷害する能力も求められている。

【００１５】

発明の要約

細胞状態特異的ＴＲＥの転写制御下のポリヌクレオチド発現を可能、又は誘導する所定の、又は特定の生理状態にある細胞に特異的な複製可能なアデノウイルスベクター、又はそれらの使用方法が提供される。これら複製可能アデノウイルスベクターでは、１またはそれ以上のアデノウイルス遺伝子が細胞特異的転写制御エレメント（ＴＲＥ）の転写制御下にある。好ましくは、細胞状態特異的ＴＲＥの転写制御下にあるアデノウイルス遺伝子は、アデノウイルスの増殖に必須な遺伝子である。細胞状態特異的ＴＲＥの制御下にあるトランス遺伝子も存在するだろう。本発明のアデノウイルスベクターでは、細胞状態特異的ＴＲＥは、異常な生理学的状態、即ち正常な生理状態では分裂していないか、又は制御された分裂を行っている同一細胞の典型的又は正常な生理学的状態からはずれている、特定の可逆性の生理学的状態にある細胞に於いて活性である。

【００１６】

従って１つの観点では、発明は細胞状態特異的ＴＲＥの転写制御下にあるアデノウイルス遺伝子を含むアデノウイルスベクターを提供する。別の実施態様では、細胞状態特異的ＴＲＥはヒト型である。別の実施態様では、細胞状態特異的ＴＲＥは細胞状態特異的プロモーター及びエンハンサーを含む。更に別の実施態様では、細胞状態特異的ＴＲＥは細胞型特異的ＴＲＥに並列しており、又２つの要素は一緒にアデノウイルス遺伝子を制御する。即ち、発明は細胞状態特異的ＴＲＥ及び細胞型特異的ＴＲＥを含むＴＲＥを具備するアデノウイルスベクターを提供する。

【００１７】

幾つかの実施態様では、細胞状態特異的ＴＲＥの転写制御下にあるアデノウイルス遺伝子は、複製に必須なアデノウイルス遺伝子である。幾つかの実施態様では、複製に必須なアデノウイルス遺伝子は初期遺伝子である。別の実施態様では、初期遺伝子はＥ１Ａである。別の実施態様では、初期遺伝子はＥ１Ｂである。更に別の実施態様では、Ｅ１Ａ及びＥ１Ｂは共に細胞状態特異的ＴＲＥの転写制御下にある。別の実施態様では、複製に必須なアデノウイルス遺伝子は後期遺伝子である。

【００１８】

別の実施態様では、細胞状態特異的ＴＲＥは低酸素反応性エレメントを含む。別の実施態様では、細胞状態特異的ＴＲＥは配列番号１のヌクレオチドを含む。

細胞状態特異的ＴＲＥは細胞周期特異的ＴＲＥを含む。細胞周期特異的ＴＲＥはＥ２Ｆ１５’フランキング領域に由来する。実施態様の一つでは、細胞周期特異的ＴＲＥは、配列番号２記載のヌクレオチド配列を含む。

【００１９】

別の実施態様では、アデノウイルスベクターは更に、遺伝子が細胞状態特異的ＴＲＥの転写制御下にあるトランス遺伝子を含む。幾つかの実施態様では、トランス遺伝子は細胞傷害性遺伝子である。

別の実施態様では、アデノウイルスベクターは第１細胞状態特異的ＴＲＥの制御下のアデノウイルス複製に必須なアデノウイルス遺伝子、及び第２細胞状態特異的ＴＲＥの制御下のアデノウイルス複製に必須な第２アデノウイルス遺伝子を具備する。第１及び第２細胞状態特異的ＴＲＥは互いに同一、実質同一、あるいは異なってもよい。

【００２０】

別の実施態様では、アデノウイルスベクターは第１細胞状態特異的ＴＲＥの制御下のアデノウイルス複製に必須なアデノウイルス遺伝子及び第２細胞状態特異的ＴＲＥの制御下にあるトランス遺伝子を含む。第１及び第２細胞状態特異的ＴＲＥは相互に実質同一、又は異なってもよい。

別の実施態様では、アデノウイルスベクターは細胞状態特異的ＴＲＥの転写制

御下にあるアデノウイルス遺伝子及び細胞型特異的ＴＲＥ転写制御下にある第２アデノウイルス遺伝子を含む。別の実施態様では、アデノウイルスベクターは細胞状態特異的ＴＲＥの転写制御下にあるアデノウイルス遺伝子及び細胞型特異的ＴＲＥの転写制御下にあるトランス遺伝子を含む。

【００２１】

別の観点では、本発明はここに記載のアデノウイルスベクターを含む宿主細胞を提供する。

別の観点では、本発明はここに記載のアデノウイルスベクターを含む医薬組成物を提供する。

別の観点では、本発明はここに記載のアデノウイルスベクターを含むキットを提供する。

【００２２】

別の観点では、細胞をここに記載のアデノウイルスに接触せしめ、それによって該ベクターが細胞に侵入することを含む、標的細胞（即ち細胞状態特異的ＴＲＥが機能することを可能にする、または誘導する細胞）内に選択的細胞傷害性を付与する方法を提供する。

別の本発明の実施態様は、その細胞の状態が細胞状態特異的ＴＲＥを機能させる、又は機能を誘導する哺乳動物細胞内にて優先的に複製するアデノウイルスである。

【００２３】

別の観点では、ここに記載のアデノウイルスベクターと、その細胞の状態が細胞状態特異的ＴＲＥの機能を可能とする哺乳動物細胞とを組み合わせることによりアデノウイルスが増殖されることを具備する方法である。その細胞状態が細胞状態特異的ＴＲＥの機能を可能にする哺乳動物細胞特異的アデノウイルス増殖法である。

【００２４】

本発明は更に、そのアデノウイルスベクターが腫瘍細胞内に入り、そして腫瘍細胞に関する選択的細胞傷害性を発揮するように本発明のアデノウイルスと腫瘍細胞とを接触せしめることを含む、腫瘍細胞、より具体的には標的腫瘍細胞（即

ち、細胞状態特異的ＴＲＥが機能することを可能にする、または誘導する腫瘍細胞）の増殖を抑制する方法を提供する。

【００２５】

別の観点では、生物学的サンプルの細胞をここに記載のアデノウイルスベクターと接触せしめ、そしてアデノウイルスの複製が有る場合にはそれを検出することを含む。その細胞状態が生物サンプル中の細胞状態特異的ＴＲＥの機能を可能にする細胞を検出するための方法を提供する。

（発明の詳細な説明）

我々は、その細胞状態が細胞状態特異的転写制御反応エレメント（ＴＲＥ）の機能を可能にする状態にある細胞に於いて、優先的に転写されるように細胞状態特異的ＴＲＥ転写制御下にあるアデノウイルス遺伝子を含んでいる複製可能アデノウイルスベクターを発見し、また構築し、そしてこれらアデノウイルスベクターを用いる方法を開発した。幾つかの好ましい実施態様では、本発明のアデノウイルスは細胞状態特異的ＴＲＥにより制御される転写に必要な転写因子及び／又は補助因子の結合によって特異的に制御されるＴＲＥの転写制御下にあるアデノウイルスの複製に必要なアデノウイルス遺伝子を少なくとも１つ、好ましくは少なくとも１個の初期遺伝子を含む。複製に必要な少なくとも１個のアデノウイルス遺伝子の細胞状態特異的転写を提供することで、本発明は選択的複製及び／又は選択的転写により特異的細胞傷害作用に関し利用できるアデノウイルスを提供する。これは的を絞った細胞殺滅が望まれる癌との関係に於いて特に有用である。これはまた、対象となる細胞の選択的破壊及び／又は抑制が望ましい、その他の非腫瘍性細胞の標的化した細胞傷害性作用に関しても有用である。このベクターは、その細胞状態が細胞状態特異的ＴＲＥの機能を可能にする細胞の存在、例えば適当な生物学的（たとえば臨床の）サンプルの検出にも有用であろう。更にアデノウイルスベクターは、細胞状態特異的ＴＲＥを利用することで標的細胞内に１またはそれ以上の所望蛋白質を随意選択的に産生できる。

【００２６】

我々は本発明のアデノウイルスが、その細胞状態が細胞状態特異的ＴＲＥを機能可能にする状態にある細胞内に於いて、細胞状態特異的ＴＲＥに選択的に作動

可能に連結したアデノウイルス遺伝子を複製及び／又は発現することを見いだした。特定の、分化した細胞にて優先的に複製することを目的とした従来報告されたアデノウイルスベクターと異なり、本発明のアデノウイルスは細胞型に特異的でない制御エレメントを含む。むしろ、それらは細胞状態特異的アデノウイルス複製及び／又はアデノウイルス遺伝子及び／又はトランス遺伝子に作動可能に連結した細胞状態特異的発現を付与する。

【００２７】

本発明のアデノウイルスベクターは、可逆的及び／又は進行的である特異的生理（即ち環境的又は代謝的）特性を有する細胞のなかで機能する細胞状態特異的ＴＲＥを含む。標的細胞は、細胞状態ＴＲＥを機能させることを目的として低酸素分圧の様な異常な生理状態を表すか、あるいは熱又は電離放射線の様な異常な環境条件下に置かれるだろう。これらベクターが複製し易いことは、必要な生理状態又は条件（例えば、異常な生理学的状態）にある細胞の複製レベルを、必要な生理状態を示さない細胞（例えば正常な生理学的条件下にある）での複製レベルと比較することで示される。即ち、本発明はアデノウイルスに関し望ましくないと考えられている観点、例えばその複製や想定される付随する免疫原性を利用し、そして利点としている。制御不能な感染の確率は、ウイルス複製に関する細胞状態特異的条件によって有意に低下している。特定の理論に結びつける意図無しに、本発明者はアデノウイルス蛋白質の産生が免疫システムの活性化及び／又は促進し、一般的及び／又は特異的に標的細胞にアデノウイルス蛋白質を産生させ、これが患者がしばしば軽度から重度の免疫抑制状態にある癌では重要であることに注目した。

【００２８】

本発明のアデノウイルスベクターは、その治療が例えば手術不能又は治療不可能な特定の癌について狙いを絞った様な特異的治療法に於いて得に有用である。本発明の重要な特徴の一つは、このベクターが癌の組織や細胞の型に関係なく治療にとって有効であることであり、そして更にそれらの細胞傷害性が特定の部位を標的化できることである。

【００２９】

一般的な技術

本発明の実施では、特記なき限り分子生物学（組換え体技術を含む）、微生物学、細胞生物学、生化学及び免疫学の通常の技術を利用しており、これらは例えば「Molecular Cloning: A Laboratory Manual」，第2版（Sanbrookら、1989）；「Oligonucleotide Synthesis」（M.J.Gait，編集，1984）；「Animal Cell Culture」（R. I. Freshney，編集、1987）；「Methods in Enzymology」（Academic Press, Inc.）；「Handbook of Experimental Immunology」（D.M.Wei & C.C.Blackwell，編集）；「Gene Transfer Vectors for Mammalian Cells」（J.M.Miller & M.P. Calos，編集、1987）；「Current Protocols in Molecular Biology」（F.M. Ausubelら、編集、1987）；「PCR: The Polymerase Chain Reaction」，（Mullisら、編集、1994）；「Current Protocols in Immunology」（J.E.Coliganら、編集、1991）の様な文献に十分説明されている。

【0030】

アデノウイルスに関する技術に関しては、特に、FelsnerとRingold（1989）「Nature」337：387－388；BernerとSharp（1983）「Nucl. Acids Res.」11：6003－6020；Graham（1984）「EMBO J.」3：2917－2922；Bettら、（1993）「J. Virology」67：5911－05921；Bettら（1994）「Proc. Natl.」Acad. Sci. USA 91：8802－8806参照。

【0031】

定義

ここに用いる「転写反応エレメント」又は「転写制御エレメント」、あるいは「ＴＲＥ」とは、ＴＲＥを機能可能にする、宿主内で作動可能に連結されたポリヌクレオチド配列の転写を増加するポリヌクレオチド配列、好ましくはＤＮＡ配列である。ＴＲＥはエンハンサー及び／又はプロモーターを含むことができる。

【００３２】

ここで用いる「細胞状態特異的ＴＲＥ」は、細胞状態特異的ＴＲＥを機能することができる細胞．即ちこれに限定されることは無いが、異常な生理状態を含む特定の生理学的条件を示す細胞内において作動可能に連結されたポリヌクレオチドに転写活性を付与するものである。「細胞状態」とは、不可逆及び／又は進行的である、細胞の所定の又は特定の生理学的状態（又は条件）を意味する。生理学的状態は内部的又は外部的に作られるだろう；例えば、それは代謝状態（低酸素の様な）であり、又はそれは熱又は電離放射線により生成されるだろう。「細胞状態」は、正常状態下で不可逆的である細胞の分化状態に関係する「細胞型」とは異なる。一般に（絶対ではない）、ここに論ずるように、細胞状態は、例えば以下に示すような異常生理状態に包含される。

【００３３】

「正常細胞状態」又は「正常生理状態」は、正常な生理学的条件にあり、そして制御された様式にて非分裂または分裂する細胞、即ち正常な生理学的状態にある細胞の状態である。

ここで交換可能に使用される「異常細胞状態」及び「異常生理状態」という用語は、異常な生理学的条件に反応する、その結果である、又は影響を受ける細胞の状態を意味する。異常細胞状態は、細胞特異的でも細胞非特異的でもない。異常細胞状態は、非分裂／制御された分裂状態、かつ正常な生理学的条件下にある同一タイプの細胞に関係して規定される。

【００３４】

「正常な生理的条件」は当業者に既知である。これら条件は、細胞の体内に於ける局在によって変わるだろう。例えば低酸素分圧は組織間で変わる。ＴＲＥが正常状態からの逸脱に反応するかどうか決定することを目的とするインビトロ分析に関し、これら条件は一般には約20%O_2の酸素濃度、及び約37℃の温度を含む

、「制御された細胞分裂」は、当業者に良く理解されている用語であり、細胞の正常な有糸分裂活性を意味する。当業者は正常な有糸分裂活性は細胞の型によって変わることを理解する。例えば、組織に於ける多くの最終分化した細胞は、殆ど有糸分裂活性を持たないか、全く持たないが、造血細胞は一般に有糸分裂活性的である。

【００３５】

ここに用いられる「異常生理条件」又は「異常生理状態」は正常生理状態から逸脱した状態を意味し、低酸素状態の様な酸素濃度の変化、生理学的温度からはずれた温度；アポトーシスを惹起する条件；電離放射線やＵＶ照射を含む放射線；例えば網膜芽腫蛋白質（Ｒｂ）の様な細胞分裂をコントロールする因子の欠損、量的不足、または不活性化の結果起こる脱制御された細胞分裂の脱制御；細胞周期のタイミングの変動；病原菌の感染；化学物質への曝露；又は上記条件の組合せにより特徴付けられる生理学的条件を含むが、これに限定されるものではない。別の例は、いずれの型の細胞にも存在でき、又は存在する、即ちその存在が細胞の分化状態に依存する、又は関係しない突然変異である。

【００３６】

ここで用いられる「標的細胞」とは、細胞が作動可能に連結されたポリペプチドの転写活性化に影響する様な細胞状態特異的ＴＲＥの機能を可能または誘導する細胞である。標的細胞は異常な生理状態であってもよい、必要な生理（又は環境）状態を表す細胞である。好ましくは標的細胞は哺乳動物細胞、好ましくはヒト細胞である。標的細胞は新生物でも、又は新生物でなくともよい。転写活性化により、標的細胞での転写のレベルは（例えば、必要生理学状態を表さないとき、一般には正常生理状態）コントロール細胞での転写レベルの少なくとも約２倍、好ましくは少なくとも約５倍、好ましくは少なくとも約１０倍、より好ましくは少なくとも約２０倍、より好ましくは少なくとも約５０倍、より好ましくは約１００倍、より好ましくは少なくとも約２００倍、より更に好ましくは少なくとも約４００倍～約５００倍、更により好ましくは少なくとも約１０００倍増加することを意味する。正常レベルは通常、細胞状態特異的ＴＲＥを活性化する条件下に試験した細胞に於ける活性のレベル（もしあれば）、又は必要生理条件を表

す細胞内にて測定された時に細胞状態特異的ＴＲＥを欠くレポーター構築体の活性のレベルである。

【００３７】

細胞状態特異的ＴＲＥの「機能的に保存された」変異体とは、別の細胞状態特異的ＴＲＥとは異なるが、それでも細胞状態細胞特異的転写活性を保持している細胞状態特異的ＴＲＥである。細胞状態特異的ＴＲＥ内の差は例えば単一塩基変異、塩基の付加、欠失、及び／又は修飾により生ずる線状配列の差に基づくことがある。この差は、糖、及び／又は細胞状態特異的ＴＲＥの塩基間の結合の変化より生じ得る。

【００３８】

「アデノウイルスベクター（ａｄｅｎｏｖｉｒｕｓ　ｖｅｃｔｏｒ）」又は「アデノウイルスベクター（ａｄｅｎｏｖｉｒａｌ　ｖｅｃｔｏｒ）」（交換し利用できる）は本発明のポリペプチド構築体を含む。本発明のポリペプチド構築体はＤＮＡ、アデノウイルスコート内に封入されたＤＮＡ、別のウイルス又はウイルス様形状内にパッケージされたＤＮＡ（たとえば、単純ヘルペス及びＡＡＶ）、リポソーム内に封入されたＤＮＡ、ポリリジンと複合したＤＮＡ、合成ポリカチオン分子と複合したＤＮＡ、トランスフェリンと接合したＤＮＡ及び分子を免疫学的に「マスク」する、及び／又は半減期を増加する化合物、たとえば、ＰＥＧと複合したＤＮＡ、及び非ウイルス蛋白質と結合したＤＮＡを含む幾つかの形状であるが、これに限定されるものではない。好ましくは、ポリヌクレオチドはＤＮＡである。ここで用いられる場合、「ＤＮＡ」は塩基Ａ、Ｔ、Ｃ及びＧだけでなく、それらの類似体または修飾型塩基、たとえば、メチル化ヌクレオチド、中間ヌクレオチド修飾体、たとえば、非荷電型結合及びチオ酸塩、糖類似体の利用、及び修飾された、及び／又は変更された主鎖構造、たとえば、ポリアミドも含む。

【００３９】

「ポリヌクレオチド」及び「核酸」という用語は、ここでは交換使用可能であるが、任意の長さを持つリボヌクレオチド又はデオキシリボヌクレオチドのいずれかである重合ヌクレオチドの形状を意味する。これら用語には、単鎖、２本鎖

、３本鎖ＤＮＡ、ゲノムＤＮＡ、ｃＤＮＡ、ＲＮＡ、ＤＮＡ－ＲＮＡハイブリッド、又はプリン及びピリミジン塩基を含むポリマー、あるいはその他の天然、化学的、生化学的に修飾された、非天然あるいは誘導化ヌクレオチド塩基を含む。ポリヌクレオチドの主鎖は、糖及びリン酸基（典型的にはＲＮＡ及びＤＮＡに見いだせる）、又は修飾もしくは置換された糖あるいはリン酸基を含むことができる。あるいは、ポリヌクレオチドの主鎖はフォスフォルアミデートの様な合成置換基のポリマーを含むことができ、従ってオリゴデオキシヌクレオシドフォスフォルアミデート（Ｐ－ＮＨ２）又は混合型フォスフォルアミデート－フォスフォジエステルオリゴマーである。Ｐｅｙｒｏｔｔｅｓら（１９９６）「Ｎｕｃｅｉｃ Ａｃｉｄｓ Ｒｅｓ．」２４：１８４１－８；Ｃｈａｔｕｒｖｅｄｉら、（１９９６）「Ｎｕｃｌｅｉｃ Ａｃｉｄｓ Ｒｅｓ．」２４：２３１８－２３；Ｓｃｈｕｌｔｚら、（１９９６）「Ｎｕｃｌｅｉｃ Ａｃｉｄｓ Ｒｅｓ．」２４：２９６６－７３。フォスフォジエステル結合の代わりにフォスフォロチエート結合が利用できる。Ｂｒａｕｎｓら、（１９８８）「Ｊ．Ｉｍｍｕｎｏｌ．」１４１：２０８４－９；Ｌａｔｉｍｅｒら、（１９９５）「Ｍｏｌ．Ｉｍｍｕｎｏｌ．」３２：１０５７－１０６４。更に、二本鎖ポリヌクレオチドは、化学的合成した単鎖ポリヌクレオチド産物から、相補鎖を合成し、適当な条件下にアニーリングすること、又は相補鎖を適当なプライマーとＤＮＡポリメラーゼを用いて新たに合成することで得られる。

【００４０】

以下は非限定的なポリヌクレオチド例である：遺伝子、又は遺伝子断片、エクソン、イントロン、ｍＲＮＡ、ｔＲＮＡ、ｒＲＮＡ、リボザイム、ｃＤＮＡ、組換え体ポリヌクレオチド、分枝型ポリヌクレオチド、プラスミド、ベクター、いずれかの配列の単離ＤＮＡ、何れかの配列の単離ＲＮＡ、核酸プローブ、及びプライマー。ポリヌクレオチドは修飾ヌクレオチド、たとえば、メチル化ヌクレオチド及びヌクレオチド類似体、ウラシル、その他の糖類及び結合基、たとえば、フルオロリボーソーム及びチオ酸塩並びにヌクレオチド分枝を含むだろう。ヌクレオチドの配列は、非ヌクレオチド成分によって中断されるだろう。ポリヌクレオチドは重合後、標識成分による複合体化等により更に修飾されるだろう。本定

義に含まれるその他のタイプの修飾は、キャップ、１又はそれ以上の天然に生ずるヌクレオチドの類似体による置換、及びポリヌクレオチドを蛋白質、金属イオン、標識成分、他のヌクレオチド、又は固相支持体に結合させるための手段の導入である。好ましくは、ポリヌクレオチドはＤＮＡである。ここで使用する場合、「ＤＮＡ」には塩基Ａ、Ｔ、Ｃ及びＧだけでなく、それらの類似体、又はそれらの修飾体、たとえば、メチル化ヌクレオチド、中間ヌクレオチド修飾体、たとえば、非電荷結合及びチオ酸塩、糖類似体の利用並びに修飾された、及び／又は変更された主鎖構造、たとえば、ポリアミドも含まれる。

【００４１】

ポリヌクレオチド又はポリヌクレオチド域は他配列に対し特定割合の「配列同一性」（例えば８０％、８５％、９０％又は９５％）を持つことは、配列をそろえ整列した場合に、その割合の塩基が比較する２配列間で同一であることを意味する。この整列及び相同性％、又は配列同一性は、例えば「Ｃｕｒｒｅｎｔ Ｐｒｏｔｏｃｏｌｓ ｉｎ Ｍｏｌｅｃｕｌａｒ Ｂｉｏｌｏｂｙ」（Ｆ．Ｍ．Ａｕｓｕｂｅｌら、１９８７）追補３０、７．７．１８章、表７．７．１に記載の様な、当業者公知のソフトウエアープログラムを利用し決定することができる。好ましい整列プログラムはＡＬＩＧＮ Ｐｌｕｓ（Ｓｃｉｅｔｉｆｉｃ ａｎｄ Ｅｄｕｃａｔｉｏｎａｌ Ｓｏｆｔｗａｒｅ，Ｐｅｎｎｓｙｌｖａｎｉａ）であり、好ましくはデフォルトパラメータを利用する。

【００４２】

「転写制御下」とは当業者公知の用語であり、ポリヌクレオチド配列、通常ＤＮＡ配列の転写が、転写の開始、又は促進に寄与するエレメントへの作動可能（動作可能に）に結合されることに依存していることを意味している。「作動可能に連結された」とは、エレメントがそれらが機能可能な配置にある並列状態を意味する。

【００４３】

「複製」及び「増殖」は交換可能に利用され、発明のポリヌクレオチド構築体の産生、または増幅の能力を意味する。この用語は当分野で公知である。本発明の目的に関しては、複製はアデノウイルス蛋白質の産生を含み、一般にはアデノ

ウイルスの産生を意味する。複製はバーストアッセイ、プラークアッセイ、又は１段階増殖曲線アッセイの様な当分野で標準の、ここに記載するアッセイにより測定できる。

【００４４】

ここで使用する場合「細胞傷害性」とは当分野で公知の用語であり、細胞の通常の生化学的又は生物学的活性が傷害される状態（即ち阻害される）を意味する。これらの活性には、代謝、細胞複製、ＤＮＡ複製、転写、分子取り込みが含まれるが、これに限定されない。「細胞傷害性」は、細胞死及び／又は細胞溶解を含む。細胞傷害性を示す、色素除外法、^{3}Ｈ－チミジン取り込み、及びプラークアッセイの様なアッセイは当分野で既知である。

【００４５】

ここで使用される場合「選択的細胞傷害性」という用語は、細胞状態特異的ＴＲＥが機能できない細胞（非標的細胞）に対する本発明のアデノウイルスベクターにより付与される細胞傷害性に比べた時に、細胞状態特異的ＴＲＥの機能が作動可能になるか、又は誘導される細胞（標的細胞）に対し本発明のアデノウイルスベクターにより付与される細胞傷害性を意味する。この様な細胞傷害性は、例えばプラークアッセイ、標的細胞を含む腫瘍の大きさの減少又は安定化により、あるいは腫瘍細胞に特徴的なマーカー、あるいは組織特異的マーカー、例えば前立腺特異抗原の様な癌マーカーの血清レベルの減少又は安定化により測定できるだろう。

【００４６】

アデノウイルスとの関係では、「異種ポリヌクレオチド」又は「異種遺伝子」または「トランス遺伝子」は、野生型アデノウイルスに存在しないポリヌクレオチド又は遺伝子である。好ましくは、トランス遺伝子もアデノウイルスベクターによる誘導前には標的細胞内に発現又は存在していないだろう。好ましいトランス遺伝子の例は以下に提供される。

【００４７】

アデノウイルスの関係では、「異種」プロモーター又はエンハンサーは、アデノウイルス遺伝子に関連しない、又は由来しないものである。

アデノウイルスの関係では、「内因性」プロモーター、エンハンサー又はＴＲＥは天然の、又はアデノウイルスに由来するものである。

細胞状態特異的ＴＲＥの関係では、「異種」プロモーター又はエンハンサーは、細胞に於いて細胞状態特異的ＴＲＥに関係又は由来しないものである。異種プロモーター又はエンハンサーの例は、アルブミンプロモーター又はエンハンサー、及び他のウイルスプロモーター及びエンハンサー、たとえば、ＳＶ４０、又は細胞タイプ特異的ＴＲＥ、たとえば、前立腺特異的ＴＲＥである。

【００４８】

「細胞タイプ特異的ＴＲＥ」は他のタイプの細胞に比べ、特異的タイプの細胞に於いて優先的に機能する。細胞状態に対し「細胞型」は不可逆的である細胞の分化状態を反映している。例えば、前立腺特異抗原は前立腺細胞に発現されるが、その他タイアの細胞、たとえば、肝細胞、星状細胞、心臓細胞、リンパ細胞等では本質的に発現されない。一般に、細胞タイプ特異的ＴＲＥは１種類のタイアの細胞で活性である。細胞タイプ特異的ＴＲＥが１種類以上のタイアの細胞で活性な場合、その活性は限られた数のタイアの細胞に限定され、即ち全てのタイアの細胞で活性であることはない。細胞タイプ特異的ＴＲＥは腫瘍細胞特異的でも、また特異的でなくともよい。

【００４９】

腫瘍増殖を「抑制する」とは、ここに記載のアデノウイルスベクターとの接触無し、即ちトランスフェクション無しの場合の増殖に比し低下された増殖状態を示す。腫瘍細胞の増殖は、腫瘍サイズ計測、^{3}Ｈ－チミジン取り込みアッセイを利用した腫瘍細胞増殖の有無の決定、又は腫瘍細胞数の測定を含む、既知の方法によりアッセイできるが、これらに限定されない。「抑制」腫瘍細胞増殖とは、以下の状態のいずれか、又は全てを意味する；腫瘍増殖の緩慢化、遅延、又は停止及び腫瘍の縮小。

【００５０】

ここで用いる場合、「新生物細胞」、「新生物」、「腫瘍」、「腫瘍細胞」、「癌」及び「癌細胞」（置き換え使用可能）という用語は、比較的に自律的増殖を示す細胞を意味しており、その結果それらの細胞は細胞増殖の制御の明瞭な消

失を特徴とする異常な増殖表現形を表す（即ち、脱制御細胞分裂）。新生物細胞は悪性でも、または良性でもよい。

【００５１】

「宿主細胞」は、本発明のアデノウイルスベクターのレシピエントになれる、またはレシピエントである個々の細胞、又は細胞培養体を含む。宿主細胞は、単一宿主細胞の後代を含むが、後代は自然、偶然又は意図的な突然変異、及び／又は変更により、元の親細胞と完全に同一である必要は無い（形態又は全ＤＮＡ相補性について）。宿主細胞は、インビボ又はインビトロにて本発明のアデノウイルスベクターによりトランスフェクト又は感染させられた細胞を含む。

【００５２】

「複製」及び「増殖」は交換可能に利用され、発明のアデノウイルスベクターの産生、または増幅の能力を意味する。この用語は当分野で公知である。本発明の目的に関しては、複製はアデノウイルス蛋白質の産生を含み、一般にはアデノウイルスの産生を意味する。複製はバーストアッセイ、プラークアッセイ、又は１段階増殖曲線アッセイの様な当分野で標準のアッセイであり、またここに記載されるアッセイにより測定できる。「複製」及び「増殖」は、ウイルス製造の工程に直接又は間接に含まれる、ウイルス遺伝子発現；ウイルス蛋白質、核酸又はその他成分の産生；ウイルス成分の完全ウイルスへのパッケージング；及び細胞融解の様な活性を含むが、これに限定されない。

【００５３】

「ＡＤＰコード配列」は、ＡＤＰ又はその機能断片をコードするポリヌクレオチドである。ＡＤＰとの関係に於いては、ＡＤＰの「機能的断片」はアデノウイルス複製に関し細胞傷害活性、特に細胞溶解を表すものである。細胞障害活性を測定する方法は当分野で公知であり、ここに記載される。

ＡＤＰポリペプチドを「コードする」ポリヌクレオチドは転写され、そして／又は翻訳されＡＤＰポリペプチド又はその断片を産生することができる。これらポリヌクレオチドのアンチセンス鎖も、この配列をコードすると言われる。

【００５４】

「ＡＤＰポリペプチド」は、ＡＤＰのアミノ酸配列（配列番号５参照）の少な

くとも１部分または１領域を含み、ＡＤＰに伴う機能、特に細胞障害性、より具体的には細胞溶解を示すポリペプチドである。ここに論ずる様に、これらの機能は当業者に既知の技術を利用して測定できる。例えばＡＤＰポリペプチドを提供するであろう保存的アミノ酸置換による特定配列変位が利用できることが理解される。

【００５５】

配列番号に「記載」されているポリペプチド配列とは、配列が配列番号内の連続配列に同一のもとのとして存在することを意味する。本用語は配列番号の一部分、又は領域、及び配列番号内に含まれる全配列を含む。

「生物学的サンプル」は、個体より得られる各種タイプのサンプルを包含しており、診断又はモニクリングアッセイに利用できる。本定義は生物学的起源の血液及びその他液体サンプル、生検検体または組織培養体、あるいはそれらに由来する細胞、及びその子孫の様な固形組織サンプルを包含する。本定義はさらに、その獲得後に、薬剤による処理、可溶化、蛋白質又はポリヌクレオチドといった特定成分の濃縮の様な操作が加えられたサンプルも含む。「生物学的サンプル」という用語は臨床サンプルを包含し、更に培養中の細胞、細胞上清、細胞溶解物、血清、血漿、生物学的液体及び組織サンプルも含む。

【００５６】

「個体」とは脊椎動物、好ましくは哺乳動物、より好ましくはヒトである。哺乳動物は、家畜、競技動物、齧歯類、霊長類、及び愛玩動物を含む。

「有効量」とは、臨床結果を含む有益な、又は所望する結果を表すのに十分な量である。有効量は１回またはそれ以上の投与により投与できる。本発明の目的に関しては、アデノウイルスベクターの有効量は、疾患状態の進行を和らげ、軽減し、安定化し、緩慢化し、あるいは遅延させるのに十分な量である。

【００５７】

細胞状態特異的ＴＲＥを含むアデノウイルスベクター

本発明は、細胞状態特異的ＴＲＥの転写制御下にあるアデノウイルスを含むアデノウイルスベクター構築体を提供する。好ましくは、アデノウイルス遺伝子は細胞傷害性（直接及び／又は間接）を付与し、より好ましくは細胞を死に至らし

める細胞傷害性を付与し、さらに好ましくはアデノウイルス複製に特異的な細胞障害性を付与する。細胞傷害性を付与する遺伝子の例は、アデノウイルス殺滅蛋白質（ＡＤＰ；以下考察）を含むが、これに限定されるものではない。アデノウイルスが標的細胞、即ち細胞状態ＴＲＥを機能できる、又は機能を誘導する細胞内での増殖に関し選択的（即ち優先的な）複製成分である場合。これら細胞はアデノウイルスの増殖により優先的に死滅する。標的細胞が選択的細胞傷害性、及び／又は細胞溶解性複製により破壊されると、アデノウイルスベクターの複製は大きく低下し、その結果無制御な感染や不所望の傍観者効果の確率が低下する。

【００５８】

インビトロ培養体は、標的細胞、例えば新生物細胞又はその他望ましくない細胞の発生（即ち存在）及び／又は再発に関し、混合体（例えば、生検又はその他適当な生物学的サンプル）をモニターするために維持されるだろう。細胞傷害性を更に確実にするために、細胞障害作用を有する１またはそれ以上のトランス遺伝子も存在し、選択的転写制御下にあるだろう。本実施態様では、高い確実性をもって標的細胞は破壊されるだろう。更に、又はあるいは、アデノウイルスベクターには、選択的転写制御なしに、あるいは制御下に細胞傷害性及び／又は細胞死を付与するアデノウイルス遺伝子（たとえば、ＡＤＰ）が含まれるだろう。

【００５９】

本発明のアデノウイルスベクターでの利用に適した細胞状態特異的ＴＲＥは、あらゆる種、好ましくは哺乳動物に由来する。細胞状態に反応し、又は関連して発現する数多くの遺伝子が報告されている。これら細胞状態関連遺伝子の何れかを利用することで、細胞状態特異的ＴＲＥを作製できるだろう。

細胞状態の例は細胞周期である。その発現が細胞周期に関連する遺伝子の例は、広く発現している増殖制御遺伝子の一つＥ２Ｆ－１であり、Ｓ期に最高転写活性を示す。Ｊｏｈｎｓｏｎら、（１９９４）「Ｇｅｎｅｓ　Ｄｅｖ．」８：１５１４－１５２５．　ＲＢ蛋白質及びその他のＢＢファミリーのメンバーは、Ｅ２Ｆ－１と特異的複合体を形成し、それによってその転写活性化能を阻害する。即ちＥ２Ｆ－１反応性プロモーターはＲＢによりダウンレギュレーションされる。多くの腫瘍細胞が破壊されたＲＢ機能を有し、これがＥ２Ｆ－１反応性プロモー

クーの脱抑制を誘導し、その結果細胞分裂が脱制御される。

【００６０】

従って、実施態様の一つでは、本発明はアデノウイルス遺伝子（好ましくは複製に必要な遺伝子）が細胞状態特異的ＴＲＥの転写制御下にあり、細胞状態特異的ＴＲＥが細胞周期活性化、又は細胞周期特異的ＴＲＥを含むアデノウイルスベクターを提供する。実施態様の一つでは、細胞周期活性化ＴＲＥはＥ２Ｆ１ＴＲＥである。実施態様の一つでは、ＴＲＥは図３及び配列番号２記載の配列を含む。

【００６１】

細胞状態により制御される別のグループの遺伝子は、その発現が低酸素状態に反応して増加するものである。ＢｕｎｎとＰｏｙｔｏｎ（１９９６）「Ｐｈｙｓｉｏｌ．Ｒｅｖ．」７６：８３９－８８５；ＤａｃｈｓとＳｔｒａｔｆｏｒｄ（１９９６）「Ｂｒ．Ｊ．Ｃａｎｃｅｒ」７４：５１２６－５１３２；ＧｕｉｌｌｅｍｉｎとＫｒａｓｎｏｗ（１９９７）「Ｃｅｌｌ」８９：９－１２。腫瘍細胞が多くの場合血管を形成する内皮細胞より早く増殖することが一因となり、多くの腫瘍では血液供給が不十分となり、その結果腫瘍内に低酸素域が生ずる。Ｆｏｌｋｍａｎ（１９８９）「Ｊ．Ｎａｔｌ．Ｃａｎｃｅｒ Ｉｎｓｔ．」８２：４－６；及びＫａｌｌｉｎｏｗｓｋｉ（１９９６）「Ｔｈｅ Ｃａｎｃｅｒ」Ｊ．９：３７－４０。低酸素反応の重要なインジケーターは、転写複合体ＨＩＦ－１又は低酸素誘導因子－１であり、血管内皮細胞因子を含むこれらは複数の遺伝子の制御域内で低酸素反応エレメント（ＨＲＥ）、及びエノラーゼ－１を含む糖分解酵素をコードするいくつかの遺伝子と相互作用する。マウスＨＲＥ配列は同定され、特性分析されている。Ｆｉｒｔｈら（１９９４）「Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ」９１：６４９６－６５００。ラットエノラーゼ－１プロモーター由来のＨＲＥはＪｉａｎｇら（１９９７）「Ｃａｎｃｅｒ Ｒｅｓ．」５７：５３２８－５３３５に記載されている。ラットエノラーゼ－１プロモーター由来のＨＲＥは図２に記載され、配列番号１として示されている。

【００６２】

従って、実施態様の一つでは、アデノウイルスベクターはＨＲＥを含む細胞状態特異的ＴＲＥの転写制御下にアデノウイルス遺伝子、好ましくは複製に必須なアデノウイルス遺伝子を含む。実施態様の一つでは、細胞状態特異的ＴＲＥは図２及び配列番号１に示すＨＲＥを含む。

別の細胞状態特異的ＴＲＥは、熱誘導（即ち熱ショク）プロモーター、及び電離放射線及びＵＶ照射を含む放射線曝露に反応するプロモーターを含む。例えば、初期増殖反応－１（Ｅｇｒ－１）遺伝子のプロモーター領域は、電離放射線照射により誘導されるエレメントを含む。Ｈａｌｌａｈａｎら、（１９９５）「Ｎａｔ．　Ｍｅｄ．」１：７８６－７９１；及びＴｓａｉ－Ｍｏｒｒｉｓら、（１９８８）「Ｎｕｃｌ．Ａｃｉｄｓ．Ｒｅｓ．」１６：８８３５－８８４６。熱誘導エレメントを含む熱誘導プロモーターが報告されている。例えば「Ｓｔｒｅｓｓ　Ｐｒｏｔｅｉｎｓ　ｉｎ　Ｂｉｏｌｏｇｙ　ａｎｄ　Ｍｅｄｉｃｉｎｅ」，Ｍｏｒｉｍｏｔｏ，　ＴｉｓｓｅｒｅｓとＧｅｏｒｇｏｐｏｕｌｏｓ，編集、Ｃｏｌｄ　Ｓｐｒｉｎｇ　Ｈａｒｂｏｒ　Ｌａｂｏｒａｔｏｒｙ　Ｐｒｅｓｓ：の中のＷｅｌｓｈ（１９９０）及びＰｅｒｉｓｉｃら（１９８９）「Ｃｅｌｌ」５９：７９７－８０６を参照。従って、幾つかの実施態様では、細胞状態特異的ＴＲＥは電離放射線照射に反応するエレメントを含む。実施態様の一つでは、このＴＲＥはＥｇｒ－１遺伝子の５’フランキング配列を含む。別の実施態様では、細胞状態特異的ＴＲＥは熱ショック反応性、又は熱誘導性エレメントを含む。

【００６３】

細胞状態特異的ＴＲＥも多量体を含むこともできる。例えば、ＨＲＥは少なくとも２，少なくとも３、少なくとも４又は少なくとも５個の低酸素反応性エレメントのクンデム列を含む。これらの多量体も異種プロモーター及び／またはエンハンサー配列を含むことができる。

細胞状態特異的ＴＲＥはサイレンサーを含んでいても、また含まなくてもよい、サイレンサーの存在（即ち、負の制御エレメント）は非許容細胞（即ち正常細胞状態）での転写（及びその結果としての複製）の遮断を支援するだろう。即ち、サイレンサーの存在は、非標的細胞でのアデノウイルスベクターの複製を効果的に阻害することで、細胞状態特異的複製を高めるだろう。あるいは、サイレン

サーの欠如は標的細胞での複製の実施を支援し、即ち標的細胞内の複製をより効率的にすることで、細胞状態特異的複製を高めるだろう。

【００６４】

別の実施態様では、アデノウイルスベクターは第１細胞特異状態ＴＲＥの制御下にあるアデノウイルスの複製に必須なアデノウイルス遺伝子、及び第２細胞特異状態ＴＲＥの制御下のアデノウイルス複製に必須な第２アデノウイルス遺伝子を含む。第１及び第２細胞状態特異的ＴＲＥは同一でも、同一でなくともよく、そして本質的に相互に同一であっても、また同一でなくともよい。「本質的に同一」とは、２つのＴＲＥ間の配列同一性に必要な程度を意味する。これらのＴＲＥ間の配列同一性の程度は少なくとも約８０％であり、好ましくは少なくとも約８５％であり、より好ましくは少なくとも約９０％であり、更により好ましくは少なくとも約９５％であり、更により好ましくは少なくとも約９８％であり、最も好ましくは１００％である。配列同一性は、即ち当業者に既知の「Ｃｕｒｒｅｎｔ Ｐｒｏｔｏｃｏｌｓ ｉｎ Ｍｏｌｅｃｕｌａｒ Ｂｉｏｌｏｇｙ」（Ｆ．Ｍ．Ａｕｓｕｂｅｌら、編集、１９８７）追補３０，７，７，１８章、表７．７．１の様な配列整列プログラムを用い、配列を比較することで決定できる。好ましい整列プログラムはＡＬＩＧＮ Ｐｌｕｓ（Ｓｃｉｅｔｉｆｉｃ ａｎｄ Ｅｄｕｃａｔｉｏｎａｌ Ｓｏｆｔｗａｒｅ，Ｐｅｎｎｓｙｌｖａｎｉａ）であり、好ましくはデフォルトパラメータを利用する。又は、ストリンジェント条件下のハイブリダイゼーションも配列同一性を示すことができる。ストリンジェント条件は当分野で既知である；例えばストリンジェント条件の例は、温度８０℃（又はそれ以上の温度）及び６×ＳＳＣ（またはそれ以下の濃度のＳＳＣ）である。その他のハイブリダイゼーション条件及びパラメーター（ストリンジェント度が高くなる順番で）は：インキュベーション温度が２５℃、３７℃、５０℃及び６８℃；緩衝液濃度１０×ＳＳＣ、６×ＳＳＣ、１×ＳＳＣ、０．１×ＳＳＣ（１×ＳＳＣは０．１５ＭのＮａＣｌ及び１５ｍＭのクエン酸緩衝液）及び等価のその他緩衝液；フォルムアミド濃度０％、２５％、５０％及び７５％；インキュベーション時間約２４時間から約５分；１、２またはそれ以上の洗浄段階；洗浄インキュベーション時間１、２又は１５分、洗浄液６×ＳＳＣ、１×ＳＳＣ

、０．１×ＳＳＣ又は脱イオン水である。

【００６５】

第１及び第２細胞条件特異的ＴＲＥが同一、又は本質的に同一であるアデノウイルス構築体は、特にこれらＴＲＥが初期遺伝子（たとえば、Ｅ１ＡやＥ１Ｂ）を制御する場合、例えば自動的な「自己崩壊」特性がウイルスにより遮断され、それにより増殖の程度を制御できる場合には、特定の観点に関し所望される、不安定性を示す。従って幾つかの実施態様では、第１及び第２細胞条件特異的ＴＲＥ、又は第１及び第２ＴＲＥ（一方が細胞条件特異的ＴＲＥである）は、互いの同一性が低い２つのＴＲＥ（即ち、より大きな配列変動及び非類似性を持つ）と比較した場合、不安定性を付与するに十分同一である。好適実施態様は、２つのＴＲＥがＥ１Ａ及びＥ１Ｂをそれぞれ制御するものである。「不安定性」とは、アデノウイルスが細胞内で複製する時アデノウイルスベクターの構造健全性が保存されていないことを意味しており、サザン分析の様な標準的技術を利用して測定することができる。別の実施態様では、第１及び第２ＴＲＥは十分多様であり、及び／又はベクターは安定な状態に置かれる（即ち、アデノウイルスベクターの構造健全性は保存されている）。

【００６６】

別の実施態様では、アデノウイルスベクターは第１細胞条件特異的ＴＲＥの制御下にあるアデノウイルス複製に必須なアデノウイルス遺伝子、及び第２細胞条件特異的ＴＲＥの制御下にあるトランス遺伝子を含む。第１及び第２細胞条件特異的ＴＲＥは本質的に同一であっても、また同一でなくても良い。

幾つかの実施態様では、細胞条件特異的ＴＲＥは、例えば別の細胞条件特異的ＴＲＥ、あるいは細胞型特異的ＴＲＥの様な別のＴＲＥと並列することができる。「並列する」とは、細胞条件特異的ＴＲＥと第２ＴＲＥが同一遺伝子を転写的に制御すること、又は少なくとも同一遺伝子に関し隣接することを意味する。これら実施態様に関し、細胞条件特異的ＴＲＥ及び第２ＴＲＥは以下を含む多くの配置のいずれでも良い（ａ）互いに隣り合う（即ち隣接する）；（ｂ）両方の５’が転写的に制御されている遺伝子に隣接している（即ち両者の間に中断配列を持つ）；（ｃ）一方のＴＲＥ５’ともう一方のＴＲＥ３’が遺伝子に隣接してい

るものであるが、これに限定されない。例えば、実施例１に記載され、及び図１に示されている様に、細胞型特異的ＴＲＥは細胞条件特異的ＴＲＥに並列することができ、作動可能に連結したアデノウイルス遺伝子の転写を制御することができる。この様な「複合」ＴＲＥを使って作動可能に連結されたポリペプチドの細胞状態及び細胞型特異的発現を可能にし、その結果有意により優れた特異性及び／又は効率性を付与することができるだろう。細胞型特異的ＴＲＥの例は以下に提供される。あるいは、「複合」ＴＲＥは、様々な、そしておそらく相乗的である細胞状態特異性を付与するのに利用できる。例えば、複合ＴＲＥは低酸素状態及び熱ショックに対する特異性を付与できるだろう。

【００６７】

実施例１は、ＨＲＥを含むＥ１Ａの上流、及び前立腺特異的ＴＲＥであるＰＳＡ－ＴＲＥ（エンハンサー配列－５３２２～－３７３８がＰＳＡ配列－５４１～＋１２に融合したものより成る；米国特許第５，８７１，７２６号；第５，６４８，４７８号参照）の複合ＴＲＥを持つアデノウイルス構築体を記載する。従って、幾つかの実施態様では本発明はＨＲＥ（好ましくは配列番号１記載の６７塩基断片を含む、又は構成される）及び前立腺細胞特異的ＴＲＥ、好ましくはＰＳＡエンハンサーを含む（好ましくは配列番号３の－５３２２～３７５８；あるいは約５０３～約２０８６（図４の約５０３～約２０８６の塩基）及びプロモーター、好ましくはＰＳＡエンハンサーとＰＳＡプロモーターを含む（配列番号３の約５２８５～約５８３６）を含むＴＲＥの転写制御下にある複製、好ましくは初期遺伝子の複製．好ましくはＥ１Ａ及びＥ１Ｂの複製に必須なアデノウイルスを含むアデノウイルスベクターを提供する。

【００６８】

当業者により容易に認識されるように、細胞特異的ＴＲＥはポリヌクレオチド配列であり、各種配列順序を越える機能を示すことができるものである。ヌクレオチド置換、付加、欠失の方法は当分野で既知であり、容易に利用できる機能アッセイ（ＣＡＴ又はルシフェラーゼレポーター遺伝子アッセイの様な）によって、熟練者は配列変異体が必要な細胞状態特異的転写機能を現しているかどうかを決定することができる。従って、本発明は置換、付加及び／又は欠失を含む、こ

こに開示された核酸配列の機能的に保存された変異体も含む。単一の理論によって拘束されることを望まないが、本発明者は特定の変化が発現レベルの増加を含む発現レベルの変化をもたらす可能性に注目した。発現レベルの変化、好ましくは発現レベルの上昇は、特定の例、進行性の強い癌や、あるいはより迅速且つ／又は攻撃的な細胞の殺滅パターンが望まれる場合（例えば個体の免疫抑制状態により）に特に望まれるだろう。

【００６９】

細胞状態特異的ＴＲＥ活性がどの様にして決定できるかの例としては、ポリヌクレオチド配列又はこれら配列のセットを当分野既知の方法、例えば化学的合成、部位特異的突然変異、ＰＣＲ及び／又は組換え体法を利用し作製することができる。試験すべき配列は適当なレポーター遺伝子、もとよりこれに限定されないが、例えばクロラムフェニコールアセチルトランスフェラーゼ（ＣＡＴ）、β－ガラクトシダーゼ（ｌａｃＺ遺伝子によりコードされる）、ルシフェラーゼ（ｌｕｃ遺伝子によりコードされる）、グリーン蛍光蛋白質、アルカリ性フォスファターゼ及び西洋ワサビペルオキシダーゼを含むベクター内に挿入される。これらベクター及びアッセイは、特に市販製品より容易に入手できる。この様に構築されたプラスミドは好適な宿主細胞内にトランスフェクトされ、リン酸カルシウム沈殿法、エレクトロポレーション法、リポソーム法（リポフェクション）及びＤＥＡＥデキストランの様な当分野で既知のトランスフェクション法を用い、推定される細胞状態特異的ＴＲＥによりコントロールされるレポーター遺伝子の発現について試験される。好適宿主細胞はＨｅｐ３Ｂ、ＨｅｐＧ２、ＨｕＨ７、ＨｕＨ１／Ｃ１２、ＬＮＣａＰ、ＨＢＬ－１００、Ｃｈａｎｇ肝臓細胞、ＭＣＦ－７、ＨＬＦ、ＨＬＥ、３Ｔ３、ＨＵＶＥＣ及びＨｅｌａを含むが、これに限定されない。試験されるＴＲＥレポーター遺伝子構築体をトランスフェクトされた宿主細胞は細胞状態（例えば異常な生理状態を生ずる）変化を生ずる状態に供する。これら条件に供されない同一細胞、即ち正常生理条件下、従って正常生理条件下にある同一細胞をコントロールとする。結果は、標準的アッセイを利用してレポーター遺伝子の発現レベルを測定することで得られる。特定状態及びコントロールの細胞間の発現の比較は、転写活性の有無を示す。「転写活性化」は以下に定

義される。

【００７０】

各種細胞状態特異的ＴＲＥ間の比較は、例えば正常及び異常生理条件下にある単一細胞株内の発現レベルを測定し、比較することで評価される。これらの比較は、可逆的環境条件（たとえば、熱）に供された単一細胞株及びそれら条件に供されない細胞内の発現レベルを測定し、比較することでもできる。細胞状態特異的ＴＲＥの絶対転写活性は標的細胞の性質、細胞状態特異的ＴＲＥの提供形式や形状、及び選択的に転写活性化されるコード配列の様な複数の要因に依存するだろう。使用する各種プラスミドのサイズを補償するため、活性はトランスフェクトされたプラスミドのモル数当たりの比活性として表現される。あるいは、転写（即ちｍＲＮＡ）レベルは標準的なノーザン分析とハイブリダイゼーション技術を利用し測定できる。トランスフェクションレベルは例えばサイトメガロウイルス（ＣＭＶ）極初期プロモーターのコントロール下にある、各種レポーター遺伝子をコードするプラスミドを共トランスフェクションすることで測定される。この分析は、負の制御域、即ちサイレンサーも示すことができる。

【００７１】

低酸素誘導の測定方法の例としては、Ｊｉａｎｇら（１９９７）「Ｃａｎｃｅｒ　Ｒｅｓｅａｒｃｈ」５７：５３２８－５３３５又はＤａｃｈｓら、（１９９７）「Ｎａｔｕｒｅ　Ｍｅｄ．」３：５１５－５２０に記載の様なアッセイを利用することができる。例えば推定ＨＲＥ又はその複数のタンデムコピーを、最少プロモーターエレメントと共に作動可能な状態で含み、検出可能又は検出可能なシグナルを提供するのに利用できる蛋白質をコードするポリヌクレオチドの転写を制御する構築体を宿主細胞内にトランスフェクトする。次に宿主細胞は正常酸素条件（例えば２０％O_2）及び各種低酸素、例えば５％、２％、１％、０．１％、又はより低いO_2に供する。次に作動可能に連結された発現産物（レポーター遺伝子）を測定する。

【００７２】

あるいは、細胞状態特異ＴＲＥ候補は、熱や電離放射線の様な必要な生理学的状態を提示する細胞に関しアデノウイルスを複製付与する能力について評価する

ことができる。このアッセイに関しては、推定細胞状態特異的ＴＲＥに作動可能に連結された複製に必須なアデノウイルス遺伝子を含む構築体は、必要な生理状態を示す細胞内にトランスフェクトされる。これらの細胞内でのウイルス複製は、例えば正常生理条件下にある細胞内（即ち、必要生理条件を提示しない細胞）でのウイルス複製と比較される。

【００７３】

Ａｄ２、Ａｄ５、Ａｄ１２及びＡｄ４０の様な各種アデノウイルス血清型が利用できる。例示を目的として、ここでは血清型Ａｄ５が示されている。

細胞状態特異的ＴＲＥが増殖に必須なアデノウイルスと共に用いられる場合、複製能は細胞状態を発現する標的細胞内に於いて優先的に達成できる。好ましくは、遺伝子はＥ１Ａ、Ｅ１Ｂ、Ｅ２又はＥ４の様な初期遺伝子である（Ｅ３はウイルス複製に必須ではない）。より好ましくは、細胞状態特異的ＴＲＥ下の初期遺伝子はＥ１Ａ及び／又はＥ１Ｂである。１つ以上の初期遺伝子を細胞状態特異的ＴＲＥの制御下に置くことができる。実施例１は、この様な構築体のより詳細な説明を提供する。

【００７４】

Ｅ１Ａ遺伝子は、ウイルス感染直後（０～２時間）且つ他のウイルス遺伝子より前に発現される。Ｅ１蛋白質はトランス作用型の正に作用する転写制御因子であり、他の初期ウイルス遺伝子Ｅ１Ｂ、Ｅ２、Ｅ３、Ｅ４及びプロモーター近接主要後期遺伝子の発現に関し必要とされる。その名称とは異なり、主要後期プロモーターにより駆動するプロモーター近接遺伝子はＡｄ５感染後短時間で発現される。Ｆｌｉｎｔ（１９８２）「Ｂｉｏｃｈｅｍ．Ｂｉｏｐｈｙｓ．Ａｃｔａ」６５１：１７５－２０８；Ｆｌｉｎｔ（１９８６）「Ａｄｖａｎｃｅｓ Ｖｉｒｕｓ Ｒｅｓｅｒｃｈ」３１：１６９－２２８；Ｇｒａｎｄ（１９８７）「Ｂｉｏｃｈｅｍ．Ｊ．」２４１：２５－３８。機能的Ｅ１Ａ遺伝子が無い場合、ウイルスＤＮＡ複製に必要な遺伝子産物が産生されないためウイルス感染は進まない。Ｎｅｖｉｎｓ（１９８９）「Ａｄｖ．Ｖｉｒｕｓ Ｒｅｓ．」３１：３５－８１。Ａｄ５Ｅ１Ａの転写開始部位はウイルスゲノム中４９８であり、またＥ１Ａ蛋白質のＡＴＧ開始部位は５６０である。

【００７５】

Ｅ１Ｂ蛋白質はトランス状態で機能し、核から細胞質への後期ｍＲＮＡの移動に必要である。Ｅ１Ｂ発現の欠失は、後期ウイルス蛋白質の発現の不良と、宿主細胞蛋白質合成の中止に至る不安定化をもたらす。Ｅ１ＢのプロモーターはＡｄ４０及びＡｄ５の宿主域内のエレメントの差を規定するものとして関係している；臨床的にはＡｄ４０はエンテロウイルスであり、一方Ａｄ５は急性の結膜炎の原因である。Ｂａｉｌｅｙ，　Ｍａｃｋａｙら、（１９９３）「Ｖｉｒｏｌｏｇｙ；１９３：６３１；　Ｂａｉｌｅｙら（１９９４）Ｖｉｒｏｌｏｇｙ２０２：６９５－７０６）。Ａｄ５のＥ１Ｂプロモーターは単一のＳｐ１に関する高親和性認識部位とＴＡＴＡボックスから成る。

【００７６】

アデノウイルスのＥ２域は７２ｋＤａのＤＮＡ結合蛋白質，８０ｋＤａ前駆体末端蛋白質、及びウイルスＤＮＡポリメラーゼを含む、アデノウイルスの複製に関連する蛋白質をコードしている。Ａｄ５のＥ２域は、それぞれ７６．０と７２．０にマッピングされるＥ２初期とＥ２後期と呼ばれる２カ所のプロモーターより、右側方向に転写される。Ｅ２後期プロモーターは感染後期に一過性に活性化し、Ｅ１Ａ転写活性化蛋白質とは無関係であり、Ｅ２初期プロモーターはウイルス複製初期に於いて極めて重要である。

【００７７】

Ｅ２後期プロモーターは反対側の鎖によりコードされている遺伝子のコード配列と重複しており、従って遺伝子操作に利用することはできない。しかし、Ｅ２初期プロモーターは反対鎖状にある３３ｋＤ蛋白質をコードする配列と数塩基しか重複していない。更にＳｐｅＩ制限酵素部位（Ａｄ５位置２７０８２）は上記３３ｋＤ蛋白質に関する停止コドンの一部であり、主要Ｅ２初期転写開始位置及びＴＡＴＡ結合蛋白質部位を、上流の転写因子結合部位Ｅ２Ｆ及びＡＴＦから隔てている。従って、ＳｐｅＩ末端を有する組換状態ＴＲＥを＋鎖のＳｐｅＩ部位に挿入すると、内因性のＡｄ５のＥ２初期プロモーターを破壊し、その結果Ｅ２転写体の細胞状態特異的発現が可能になるだろう。

【００７８】

Ｅ４遺伝子は複数の転写産物を有する。Ｅ４域はウイルスゲノムＤＮＡの複製の刺激と、後期遺伝子発現の刺激に関する２つのポリペプチドをコードしている。オープンリーディングフレーム（ＯＲＦＳ）３及び６の蛋白質産物は共にＥ１Ｂ及びＥ２Ｆ－１とＤＰ－１のヘテロダイマー由来の５５ｋＤ蛋白質の結合によりこれら機能を実施する。ＯＲＦ６蛋白質はその活性に関しＥ１Ｂ５５ｋＤ蛋白質との相互作用を必要とするが、ＯＲＦ３蛋白質は必要としない。ＯＲＦ３及びＯＲＦ６由来の機能蛋白質がない場合、プラークは野生型ウイルスの効率の10^{-6}低い効率で産生される。必要生理条件又は状態を示す細胞にウイルス複製を更に制限するためには、Ｅ４ＯＲＦ１～３を削除してウイルスＤＮＡ複製及び後期遺伝子合成をＥ４ＯＲＦ６蛋白質に依存させることができる。この様な変異体をＥ１Ｂ域が細胞状態特異的ＴＲＥによって制御されている配列と組み合わせることにより、Ｅ１Ｂ機能とＥ４機能の両方が細胞状態特異的ＴＲＥにより駆動しているＥ１Ｂに依存するウイルスを得ることができる。

【００７９】

本発明に関する主要遺伝子は、遺伝子Ｌ１、Ｌ２、Ｌ３、Ｌ４及びＬ５であり、これらはアデノウイルスの蛋白質をコードしている。これら遺伝子は全て（典型的には構造蛋白質をコードする）はアデノウイルス複製に必要であろう。後期遺伝子は全てが、＋５９８６から＋６０４８に局在するＡｄ５主要後期プロモーター（ＭＬＰ）の制御下にある。

【００８０】

必要とされる生理状態（例えば低酸素条件の様な異常生理状態）を示す細胞内の優先複製能の特性により選択的細胞傷害性及び／又は細胞溶解活性が付与されることに加え、本発明のアデノウイルスベクターは更に細胞状態特異的ＴＲＥの制御下にある異種遺伝子（トランス遺伝子）を含むことができる。この様にして各種遺伝的能力が細胞、特に癌細胞内に導入されるだろう。例えば、ある例では、例えば癌性の標的細胞の相対的な反応特性又は特定の攻撃性に基づき、細胞障害活性の強さ及び／又は割合を高めることが望ましいだろう。これは、細胞状態特異的複製細胞障害活性と例えばＨＳＫ－ｔｋ及び／又は５－フルオロシトシン（５－ＦＣ）を化学療法剤である５－フルオロウラシル（５－ＦＣ）に代謝する

能力を細胞に付与するシトシンデアミナーゼ（ｃｄ）の細胞特異的発現とを組み合わせることで達成できる。これらのトランス遺伝子の使用も傍観者効果を付与し得る。

【００８１】

アデノウイルスベクターを介し導入される他の望ましいトランス遺伝子には、ジフテリア毒素のＡ鎖、リシン、又はアブリンの様な細胞傷害性蛋白質をコードする遺伝子（Ｐａｌｍｉｔｅｒら、（１９８７）「Ｃｅｌｌ」５０：４３５；Ｍａｘｗｅｌｌら（１９８７）「Ｍｏｌ．Ｃｅｌｌ．」Ｂｉｏｌ．７：１５７６；Ｂｅｈｒｉｎｇｅｒら（１９８８）「Ｇｅｎｅｓ」Ｄｅｖ．２：４５３；Ｍｅｓｓｉｎｇら（１９９２）「Ｎｅｕｒｏｎ」８：５０７；Ｐｉａｔａｋら（１９８８）「Ｊ．Ｂｉｏｌ．Ｃｅｈｍ．」２６３：４９３７；Ｌａｍｂら（１９８５）「ｅｕｒ．Ｊ．Ｂｉｏｃｈｅｍ．」１４８：２６５；Ｆｒａｎｋｅｌら、（１９８９）「Ｍｏｌ．Ｃｅｌｌ．」Ｂｉｏｌ．９：４１５）、アポトーシスを開始できるファクターをコードする遺伝子、その他の能力の中で構造蛋白質の様な増殖に必須な蛋白質又は転写因子；その病原菌が細胞内で増殖する場合のウイルス又はその他病原性蛋白質をコードしているｍＲＮＡに対するアンチセンス転写体又はリボザイム；ヌクレアーゼの加工された細胞質変異体（例えばＲＮａｓｅＡ）又はプロテアーゼ（例えばアスウイン、パパイン、プロテアーゼＫ、カルボキシペプチダーゼ等）又はＰａｓ遺伝子等をコードする遺伝子が含まれる。対象となるその他遺伝子には、ＩＬ－１、－２、－６、－１２、ＧＭ－ＣＳＦ、Ｇ－ＣＳＦ、Ｍ－ＣＳＦ、ＩＦＮ－α、－β、－γ、ＴＮＦ－α、－β、ＴＧＦ－α、－β、ＮＧＦ等の様なサイトカイン、抗原、膜貫通蛋白質等が含まれる。正のエフェクター遺伝子はより初期に利用することができ、複製により細胞障害活性を示す。

【００８２】

ある実施態様では、Ｅ３域内にコードされるアデノウイルス殺滅蛋白質（ＡＤＰ）がアデノウイルスベクター内に維持される。主要後期プロモーター（ＭＬＰ）の制御下にあるＡＤＰ遺伝子は、宿主細胞溶解を実行する上で重要な蛋白質（ＡＤＰ）をコードすると考えられている。Ｆｏｌｌｅｆｓｏｎら（１９９６）「

J. Virol.」70(4):2296; Tollefsonら(1992)「J. Virol」66(6):3633。即ちADP遺伝子を含むアデノウイルスベクターは、アデノウイルスをより強力にし、より治療効果的にし、そして/又は投与条件をより低くする。

【0083】

従って、本発明はここに記した様な、更にADPをコードするポリヌクレオチド配列を含むアデノウイルスを提供する。ADPをコードするDNA配列及びADPのアミノ酸配列は図9に記される。要約すると、ADPをコードする配列は、好ましくはPCRの様な当分野で既知の技術を利用してAd2(この株のADPがより完全に特性分析されていることから)より得られる。好ましくはYリーダー(後期遺伝子の正確な発現に重要な配列)も得て、ADPコード配列と連結される。続いてADPコード配列(Yリーダー有り又は無しで)をアデノウイルスゲノム、例えばE3域(ADPコード配列がMLPにより作動される)内に導入することができる。ADPコード配列はE4域の様なアデノウイルスゲノムの別の場所に挿入することもできる。あるいは、ADPコード配列は、もとよりこれに限定されないが別のウイルスプロモーター、低酸素反応エレメントの様な細胞状態特異的TRE、又は癌胚性抗原(CEA)、ムチン、及びラットプロバシン遺伝子より得られる様な細胞型特異的TREを含む異種プロモーター(エンハンサー有り、又は無し)と作動可能に連結することもできるだろう。

【0084】

細胞型特異的エレメントを更に含む本発明のアデノウイルスベクター

必要な生理状態を提示する細胞内での、選択的複製能及び/又は細胞障害性因子をコードする遺伝子の選択的転写の特性による選択的細胞傷害性及び/又は細胞溶解活性の付与に加え、本発明のアデノウイルスベクターは細胞型特異的TREの制御下にあるアデノウイルス遺伝子及び/又は異種遺伝子(トランス遺伝子)を含むことができる。この様にして細胞傷害性はさらに特定の型の細胞に限定される。

【0085】

例えば、前立腺細胞内にて選択的に機能するTREには前立腺特異的抗原遺伝

子（ＰＳＡ－ＴＲＥ）（米国特許第５，６４８，４７８号）、腺性カリクレイン－１遺伝子（ヒト遺伝子、ｋＨＬＫＪ２－ＴＲＥ由来）及びプロバシン遺伝子（ＰＢ－ＴＲＥ）（国際特許出願番号ＰＣＴ／ＵＳ９８／０４１３２）由来のＴＲＥが含まれるが、これに限定されない。これら遺伝子は全て前立腺細胞内で優先して発現され、その発現はアンドロゲン誘導性である。一般に、アンドロゲン誘導に反応する遺伝子の発現はアンドロゲン受容体（ＡＲ）の存在を必要とする。

【００８６】

ＰＳＡは正常、過形成の及び悪性化前立腺上皮細胞により占有的に合成される；従ってその組織特異的発現は、良性前立腺肥大（ＢＰＨ）及び前立腺癌（ＣａＰ）の優れた生体マーカーである。ＰＳＡの正常血清値は通常は５ｎｇ／ｍｌ以下であり、ＢＰＨ又はＣａＰの出現に伴い増加する。Ｌｕｎｄｗａｌｌら（１９８７）「ＦＥＢＳ Ｌｅｔｔ．」２１４：３１７；Ｌｕｎｄｗａｌｌ（１９８９）「Ｂｉｏｃｈｅｍ．Ｂｉｏｐｈｙｓ．Ｒｅｓ．Ｃｏｍｍ．」１６１：１１５１；及びＲｉｅｇｍａｎｎら（１９９１）「Ｍｏｌｅｃ．Ｅｎｄｏｃｒｉｎ」５：１９２１。

【００８７】

特に前立腺細胞にて、アンドロゲンに依存した細胞特異性を提供するために用いられるＰＳＡ遺伝子の領域は、約6.0キロベースを含む。Schuurら、(1996)「J. Biol. Chem.」271:7043-7051。ヒトでの約1.5kbのエンハンサー域は、ＰＳＡ遺伝子の転写開始部位に対しｎｔ－５３２２～ｎｔ－３７３８の範囲に局在する。これら２つの遺伝的要素が並列すると、完全に機能的な、最低限の前立腺特異的エンハンサー／プロモーター（ＰＳＥ）ＴＲＥができる。ＰＳＡの約６．０ｋｂの領域である別の部分は、必要な機能性が維持できる範囲に於いて本発明に利用できる。実施例１では、ＨＲＥ及びＰＳＡ－ＴＲＥを含む複合ＴＲＥ、－５４１～＋１２のＰＳＡプロモーターと融合した－５３２２～３７７８のＰＳＡエンハンサーを含むＰＳＡ－ＴＲＥを具備するアデノウイルスベクターＣＮ７９６が記述されている。このＰＳＡ－ＴＲＥは、アデノウイルスベクターＣＮ７０６に由来する。Ｒｏｄｒｉｇｕｅｚら（１９９７）「Ｃａｎｃｅｒ Ｒｅｓｅａｒｃｈ」５７：２５５９－２５６３。従って、実施態様の一つでは、アデノウイルス

ベクターは細胞状態特異的ＴＲＥ、ＨＲＥ及び細胞型特異的ＴＲＥであるＰＳＡ－ＴＲＥを含む複合ＴＲＥの転写制御下にあるアデノウイルスＥ１Ａ遺伝子を含む。

【００８８】

本発明に用いられるＰＳＥ及びＰＳＡ　ＴＲＥは図４（配列番号３）に記載の配列に由来する。エンハンサーエレメントは図４（配列番号３）の約５０３～約２０８６のヌクレオチドである。プロモーターは図４（配列番号３）の約５２８５～約５８３６のヌクレオチドである。従って、幾つかの実施態様では、複合ＴＲＥは配列番号１を含むＨＲＥを含み、ＰＳＡ－ＴＲＥは配列番号３の約５０３～約２０８６のヌクレオチドを含む。別の実施態様では、複合ＴＲＥは配列番号１を含むＨＲＥを具備し、ＰＳＡ－ＴＲＥは配列番号３の約５０３～約２０８６のヌクレオチド及び配列番号３の約５２８５～約５８３６のヌクレオチドを含む。上記の如く、これら複合（ＨＲＥ／ＰＳＡ）ＴＲＥは作動可能に複製に必須なアデノウイルス遺伝子、特にＥ１Ａ又はＥ１Ｂの様な初期遺伝子と連結している。実施例１はこの様な構築体を記述している。

【００８９】

本発明では、細胞状態特異的ＴＲＥ及び細胞型特異的ＴＲＥを含む複製可能アデノウイルスベクターが、優先的に特定の主要細胞内にて機能する細胞型特異的ＴＲＥを利用し得る。主要細胞特異的ＴＲＥの非限定的例及び潜在的標的細胞の非限定例には、以下の遺伝子のＴＲＥが含まれる；αフェト蛋白質（ＡＦＰ）（肝臓癌）、ムチン様糖蛋白質ＤＦ３（ＭＵＣＩ）（乳癌）、癌胚性抗原（ＣＥＡ）（直腸結腸癌、胃癌、膵臓癌、乳癌及び肺癌）、プラスミノーゲン活性化ウロキナーゼ（ｕＰＡ）及びその受容体（乳癌．結腸癌、及び肝臓癌）、ＨＥＲ－２／ｎｅｕ（ｃ－ｅｒｂ２／ｎｅｕ）（乳癌、卵巣癌、胃癌、及び肺癌）。

【００９０】

その他の細胞タイプ特異的ＴＲＥは以下に例示する遺伝子に由来する（ＴＲＥが特異的に機能している細胞の型をカッコ内に示す）：血管内皮増殖因子受容体（内皮細胞）、アルブミン（肝臓）．第ＶＩＩ因子（肝臓）、脂肪酸合成酵素（肝臓）、フォンウイルブランド因子（脳上皮細胞）、アルファアクチン及びミオ

シン重鎖（共に平滑筋）、合成酵素Ｉ（小腸）、Ｎａ－Ｋ－Ｃｌトランスポーター（腎臓）。その他細胞タイプ特異的ＴＲＥは当分野で既知である。

【００９１】

ＡＦＰは癌胎性蛋白質で、その発現は一次的には内胚葉性起源の組織の発生に限定される（卵黄嚢、胎児肝臓、及び食道）が、その発現レベルは組織及び発生段階によって大きく変動する。ＡＦＰは、その血清濃度が多くの肝臓癌患者で上昇し、進行した病気を持つ患者ほどＡＦＰのレベルが高いことから臨床の関心を得ている。患者の血清ＡＦＰ値は肝臓細胞癌でのＡＦＰの発現により制御されており、その周辺組織では発現していないと考えられている。即ちＡＦＰ遺伝子は肝臓癌細胞特異的に発現するよう制御されていると思われる。

【００９２】

ＡＦＰ遺伝子由来の細胞型特異的ＴＲＥが特定されている。例えば、ヒトＡＦＰ特異的エンハンサー活性のクローニングと特性分析はＷａｔａｎａｂｅら（１９８７）「Ｊ．　Ｂｉｏｌ．　Ｃｈｅｍ．」２６２：４８１２－４８１８に記述されている。全５’ＡＦＰフランキング域（プロモーター、推定サイレンサー、及びエンハンサーエレメントを含む）は、転写開始部位上流約５ｋｂ内に含まれる。

【００９３】

ヒトのＡＦＰエンハンサー域は、ＡＦＰ遺伝子の転写開始部位に対し約ｎｔ－３９５４～約ｎｔ－３３３５の範囲に局在している。ヒトＡＦＰプロモーターは約ｎｔ－１７４～約ｎ＋２９の領域を包含する。これら２種類の遺伝的エレメントを並列させると、完全に機能的なＡＦＰ－ＴＲＥができる。Idoら(1995)はＨＣＣに特異的である２５９ｂｐ（ｎｔ－２３０からｎｔ＋２９）のプロモーター断片を報告している。「Ｃａｎｃｅｒ　Ｒｅｓ．」５５：　３１０５－３１０９。ＡＦＰエンハンサーは、転写開始部位に対しｎｔ－３９５４～ｎｔ－３３３５の範囲に位置する、Ａ及びＢと称される２つの領域を含む。プロモーター領域は典型的なＴＡＴＡ及びＣＡＡＴボックスを含む。好ましくは、ＡＦＰ－ＴＲＥは少なくとも１つのエンハンサー領域を含む。より好ましくは、ＡＦＰ－ＴＲＥは両方のエンハンサー領域を含む。

【００９４】

ＡＦＰ－ＴＲＥｓを含むアデノウイルスベクターに好適な標的細胞は、ＡＦＰ－ＴＲＥを機能させるいずれかの細胞である。好ましくは、これに限定されるものえはないが、ＡＦＰを発現している腫瘍細胞を含む、ＡＦＰを発現又は産生する細胞である。この様な細胞の例は肝細胞癌細胞、生殖腺及びその他生殖細胞癌（特に内胚葉洞腫瘍）、脳腫瘍細胞、卵巣腫瘍細胞、膵臓の腺房細胞癌（Ｋａｗａｍｏｔｏら、１９９２）「Ｈｅｐａｔｏｇａｓｔｒｏｅｎｔｅｒｌｏｇｙ」３９：２８２－２８６）、原発性胆嚢癌（Ｋａｔｓｕｒａｇｉら（１９８９）「ｒｉｎｓｋｏ　Ｈａｓｈａｓｅｎ」３４：３７１－３７４）、子宮内皮腺癌細胞（Ｋｏｙａｍａら、（１９９６）「Ｊｐｎ．　Ｊ．　Ｃａｎｃｅｒ　Ｒｅｓ．」８７：　６１２－６１７）及び前記の転移である（胚、副腎、骨髄、及び／又は膵臓に起こる）。幾つかの例では、特定の膵臓癌及び胃癌から肝臓への転移性疾患がＡＦＰを産生する。特に良く起こるのは肝細胞癌細胞及びその転移である。ＡＦＰ産生はＡＦＰ蛋白質産生のレベルを決定するＲＩＡ、ＥＬＩＳＡ又はウエスタンブロット、又はＡＦＰｍＲＮＡ産生のレベルを決定するノーザンブロットの様な当分野で標準のアッセイを用いて測定することができる。あるいはこれら細胞は、転写的にＡＦＰ－ＴＲＥを活性化できる（即ち、ＡＦＰ－ＴＲＥを機能化できる）それらの能力によって特定し、又は特徴付けることができる。

【００９５】

蛋白質ウロキナーゼプラスミノーゲン活性化因子（ｕＰＡ）及びその細胞表面受容体であるウロキナーゼプラスミノーゲン活性化因子受容体（ｕＰＡＲ）は最も頻繁に新生物が発生する多くに於いて発現し，癌転移に於ける代表的な重要蛋白質と考えられている。いずれの蛋白質も乳癌、結腸癌、前立腺癌、肝臓癌、腎臓癌，肺癌及び卵巣癌に関係している。ｕＰＡ及びｕＰＡＲ転写を制御する転写制御エレメントはよく研究されている。Ｒｉｃｃｉｏら、（１９８５）「Ｎｕｃｌｅｉｃ　Ａｃｉｄｓ　Ｒｅｓ．」１３：　２７５９－２７７１；　Ａｃｎｎｉｏら、（１９９１）「Ｎｕｃｅｌｉｃ　Ａｃｉｄｓ　Ｒｅｓ．」１９：　２３０３－２３０８。

【００９６】

ＣＥＡは、直腸結腸、胃及び膵臓癌の様な胃腸管及び乳癌や肺癌の様な腺腫といった内胚葉性新生物に存在する１８０，０００グルトンの糖蛋白腫瘍関連抗原である。ＣＥＡは、血中ＣＥＡが多くのＣＥＡ陽性腫瘍患者で検出されることから、臨床的に注目されている。肺癌では、全例の約５０％が血中にＣＥＡを持っており、腺癌でしばしば高濃度のＣＥＡ（２０ｎｇ／ｍｌ以上）が検出される。胃癌患者の約５０％が血清学的にＣＥＡ陽性である。

【００９７】

ＣＥＡ遺伝子の５’上流側のフランキング配列が細胞特異活性を付与することが示されている。遺伝子の５’フランキング域内の翻訳開始位置の約最初の２４メクレオチド上流にあるＣＥＡプロモーター域が、この領域が非増殖性のＨｅＬａ細胞に比べＣＥＡ産生細胞により強いプロモーター活性を提供する場合に、細胞特異性を付与することが示されている。Schreweら(1990)「Mol.Cell.Biol.」10:2738-2748。更に、細胞特異的エンハンサー域も見つかっている、ＷＯ／９５／１４１００号。全５’ＣＥＡフランキング域（プロモーター、推定サイレンサー及びエンハンサーエレメントを含む）は転写開始部位から約１，４５ｋｂ上流までに含まれていることが示されている、Ｒｉｃｈａｒｄｓら、（１９９５）；ＷＯ９５／１４１００号、Ｒｉｃｈａｒｄｓら（１９９５）によるＣＥＡ遺伝子のフランキング域に関する詳しい特性分析からは、－１３．６から－１０．７ｋｂまたは－６．１から－４．０ｋｂまでの２種類の上流域を多量体化したプロモーターに連結すると、ＣＥＡ産生ＬｏＶｏ及びＳＷ１４６３細胞にレポーター構築体の高レベル且つ高選択的な発現をもたらすことが示された。Ｒｉｃｈａｒｄｓらは（1995）更にプロモーター域が転写開始部位に対しｎｔ－９０～ｎｔ＋６９に局在し、ｎｔ－４１～ｎｔ－１８の領域が発現に必須であることした。ＷＯ９５／１４１００号は、細胞特異的活性を付与する一連の５’フランキングＣＥＡ断片、例えば約ｎｔ－２９９～約ｎｔ＋６９；約ｎｔ－９０～約ｎｔ＋６９；ｎｔ－１４，５００～ｎｔ－１０，６００；ｎｔ－１３，６００～ｎｔ－１０，６００，ｎｔ－６１００～ｎｔ－３８００を記載している。更に、細胞特異的転写活性は配列番号６に記載のｎｔ－４０２～ｎｔ＋６９のＣＥＡ断片により作動可能に連結された遺伝子に付与される。本発明に用いられているＣＥＡ－ＴＲＥ

はこれに限定されるものではないが、ヒト細胞を含む哺乳動物細胞に由来する。即ち、いずれのＣＥＡ－ＴＲＥも必要とされる所望機能性がアデノウイルスベクター内に提示される限りに於いて本発明に利用できるだろう。ＣＥＡ配列のクローニング及び特性解析は文献に記載されており、従って本発明の実施に利用可能であり、ここに詳細に記載する必要はない。

【００９８】

ＭＵＣ１遺伝子の蛋白質産物（ムチン又はＭＵＣ１蛋白質；エピシアリン；多形性上皮性ムチン又はＰＥＭ；ＥＭＡ；ＤＦ３抗原；ＮＰＧＰ；ＰＡＳ－Ｏ；又はＣＡ１５．３抗原として知られる）は通常主に胃、膵臓、肺、気管、腎臓、子宮、唾液腺、及び乳腺の腺又は管を裏打ちする上皮細胞の頂端表面に発現している。Zotterら、(1988)「Cancer Rev.」11-12:55-101;及びGirlingら、(1989)「Int. J. Cancer」43:1072-1076。しかし、ムチンはヒト乳癌の７５～９０％で過剰発現されている。Kufeら、(1984)「Hybridoma」3: 223-232。レビューに関してはHilkens(1988)「Cacner Rev.」11-12: 25-54;及びTaylor-Papadimitriouら(1990)「J. Nucl. Med. Allied Sci.」34:144-150を参照。ムチン蛋白質の発現は乳癌の分化度に相関する。Lundyら、(1985)「Breast Cancer Re/ Treat.」5:269-276。この過剰発現は転写レベルで制御されていると考えられている。

【００９９】

ヒト乳癌細胞ＭＣＦ－７及びＡＲ－７５－１でのＭＵＣ１遺伝子の過剰発現は、転写レベルで制御されていると考えられている。Kufeら(1984)；Kovarik(1993)「J.Biol.Chem.」268:9917-9926；及びAbeら(1990)「J.Cell.Physiol.」143,226-231。細胞特異的転写に関係すると思われるＴＲＥを含む転写開始部位上流約０．９ｋｂを含むＭＵＣ１遺伝子の制御配列がクローン化された。Abeら(1993)「Proc.Natl.Acad.Sci.USA」90:282-286;Kovarik(1993);及びKovarikら(1996)「J.Biol.Chem.」271:18140-18147。

【０１００】

本発明に用いられるＭＵＣ１－ＴＲＥは、ヒトに限定されないが、ヒト細胞を含む哺乳動物細胞に由来する。好ましくはＭＵＣ１－ＴＲＥはヒトのものである。実施態様の一つではＭＵＣ１－ＴＲＥはＭＵＣ１遺伝子の全０．９ｋｂ５’フ

ランキング配列を含むことができる。他の態様ではＭＵＣ１－ＴＲＥは以下の配列を含む（ＭＵＣ１遺伝子の転写開始部位に対し）：約ｎｔ－７２５～約ｎｔ＋３１、ｎｔ－７４３～約ｎｔ＋３３、ｎｔ－７５０～約ｎｔ＋３３及びｎｔ－５９８～約ｎｔ＋４８５（プロモーターに作動可能に連結された）。

【０１０１】

ｃ－ｅｒｂＢ２／ｎｅｕ遺伝子（ＨＥＲ－２／ｎｅｕ又はＨＥＲ）は１８５ｋＤの上皮増殖因子受容体関連膜貫通糖蛋白質をコードする形質転換遺伝子である。ヒトでは、ｃ－ｅｒｂＢ２／ｎｅｕ蛋白質は胎児発生中に発現されるが、成体では多くの正常組織の上皮に弱く検出できる（免疫組織的に）のみである。ｃ－ｅｒｂＢ２／ｎｅｕ遺伝子の増幅、及び／又は過剰発現が乳癌、卵巣癌、子宮癌、前立腺癌、胃癌及び肺癌を含む多くの癌に関連している。ｃ－ｅｒｂＢ２／ｎｅｕ蛋白質の過剰発現の臨床予後については乳癌及び卵巣癌で研究されている。ｃ－ｅｒｂＢ２／ｎｅｕ蛋白質の過剰発現は乳癌の管内癌の２０～４０％及び卵巣癌の３０％に起こっており、両疾患の亜分類に於ける予後不良と関係している。ヒト、ラット、及びマウスのｃ－ｅｒｂＢ２／ｎｅｕＴＲＥは同定されており、ｃ－ｅｒｂＢ２／ｎｅｕを発現細胞に特異活性を付与することが示されている。Talら、(1987)「Mol. Cell. Biol.」7:2597-2601; Hudsonら、(1990)「J.Biol.Chem.」265: 4389-4393; Grootheclaesら(1994)「Cancer Res.」54; 4193-4199; Ishiiら(1987)「Proc. Natl. Acad. Sci. USA」84:4373-4378; Scottら(1994)「J. Biol.Chem.」269:19848-19858。

【０１０２】

上記の細胞タイプ特異的ＴＲＥは本発明に於いて機能するＴＲＥの非限定的な例として提供されている。その他の細胞特異的ＴＲＥは当分野で既知であり、問題のＴＲＥの細胞特異性を同定し、試験する方法も同様に当分野で既知である。

本発明のアデノウイルスの使用

アデノウイルスは裸のポリヌクレオチド（通常ＤＮＡ）構築体；カチオン性リポソーム又はポリリジンの様なその他のカチオン性化合の様な物細胞内への侵入を促進する作用物質と複合化したポリヌクレオチド；感染性アデノウイルス粒子にパッケージされた形（アデノウイルスベクターをより免疫原性にする）；ＨＳ

Ｖ又はＡＶＶの様なその他の粒子状ウイルス形状にパッセージした形；免疫反応を高める、又は弱化する作用物質（ＰＥＧの様な）との複合化；インビトロのトランスフェクションを促進するＤＯＴＭＡ（商標）、ＤＯＴＡＰ（商標）及びポリアミンの様な作用物質と複合化したものを含む様々な形で使用できるが、これに限定されない。この様に、本発明は細胞状態産生細胞にて好ましく増幅できるアデノウイルスも提供する。「優先的に複製する」とは、アデノウイルスが必要とされる生理状態を提示しない細胞に比べ、その状態を提示する細胞にてより複製することを意味する。好ましくは、アデノウイルスは少なくとも約２倍以上複製し、好ましくは少なくとも約５倍以上、より好ましくは少なくとも約１０倍以上、さらにより好ましくは少なくとも約５０倍以上、さらにより好ましくは少なくとも約１００倍以上、さらに好ましくは少なくとも約４００倍以上～約５００倍以上、さらにより好ましくは少なくとも約１０００倍以上、最も好ましくは少なくとも１／１０^{6}以上複製する。最も好ましくは、アデノウイルスは必要とされる生理状態を提示する細胞のみにて複製する（即ち、必要とされる生理状態を提示しない細胞では複製しないか、極めてゆっくり複製する）。

【０１０３】

アデノウイルスベクターがアデノウイルス内にパッケージされる場合、標的化を高めるためにアデノウイルス自体も選別されるだろう。例えば、アデノウイルスファイバーは親和性を補助する細胞受容体との一次接触を伝達する。Ambergら、(1997)「Virol.」227:239-244参照。アデノウイルスの特定の亜属が標的型細胞に対する親和性を提示し、及び／または非標的型細胞に対する親和性を下げる場合、この様な亜属（又は亜属群）は細胞傷害性及び／又は細胞溶解の細胞特異性を更に高めるのに使用できるだろう。

【０１０４】

アデノウイルスベクターはリポソーム、当分野で公知の一般的なトランスフェクション法（たとえば、リン酸カルシウム沈殿法又はエレクトロポレーション法）、直接注入、及び静脈注入を含む各種方法により標的細胞に供給できるが、これらに限定されない。供給方法は具体的なアデノウイルスベクター（その形状を含む）及び標的細胞の型と局在（即ち、細胞がインビトロかインビボか）に大き

く依存している。

【０１０５】

パッケージされたアデノウイルスとして用いる場合、アデノウイルスベクターは約10^{4}～約10^{15}の範囲の適当な生理学的に受け入れ可能な担体の中に投与されるだろう。感染の多重度は一般に約０．００１～１００の範囲であろう。ポリヌクレオチド構築体として（即ち、ウイルスとしてパッケージされていない）投与される場合には約０．０１μｇ～１０００μｇのアデノウイルスベクターが投与できる。アデノウイルスベクターは、その目的とする応用及び宿主の免疫反応能により１回又はそれ以上投与され、複数回の同時投与として投与されることもあるだろう。免疫反応が望ましくない場合には、免疫反応は各種免疫抑制剤を利用することで除かれるため、強い免疫反応なしに繰り返し投与することができるだろう。ＨＳＶの様な別のウイルス形状にパッケージされる場合、投与量は具体的なウイルスに関する標準的知識に基づき（例えば出版された文献より容易に得られる）、そして経験的に決定することができる。

【０１０６】

本発明のアデノウイルスベクターを含む宿主細胞

本発明は、ここに記したアデノウイルスベクターを含む（すなわち、トランスフォームされた）宿主細胞も提供する。宿主細胞内での維持に必要とされる配列、例えば適当な複製起源が存在する限り、原核細胞及び真核細胞宿主細胞の両方を使用することができる。便利のために、選択可能マーカーも提供される。原核細胞宿主細胞には細菌細胞、例えば大腸菌、マイコバクテリアが含まれる。真核宿主細胞は酵母、昆虫、鳥類、植物及び哺乳動物である。宿主システムは当分野で既知であり、従ってここに記載する必要はない。

【０１０７】

本発明の組成物

本発明は更に、ここに記載のアデノウイルスベクターを含む医薬品組成物を含む組成物も提供する。この様な組成物（特に医薬品組成物）は例えば個体中での細胞殺滅の誘導及び効果の大きさを測定する場合の様な、インビトロでの投与に有用である。医薬品として受け入れ可能な成分（一般にはアデノウイルスベクタ

一の有効量）は、個体への全身投与に好適な単位投与形状、滅菌された非経口投与液または懸濁液、滅菌された経口投与液、又は経口溶液又は懸濁液、水中油型、又は油中水型乳剤等である。非経口及び経口薬物供給に関する調剤は当分野で既知であり、「Ｒｅｍｉｎｇｔｏｎ'ｓ Ｐｈａｒｍａｃｅｕｔｉｃａｌ Ｓｃｉｅｎｃｅｓ」１９ｔｈ Ｅｄｉｔｉｏｎ，Ｍａｃｋ Ｐｕｂｌｉｓｈｉｎｇ（１９９５）に記載されている。医薬品組成物は更に本発明のアデノウイルスベクターの凍結乾燥された及び／又は再構築された形状（アデノウイルスの様なウイルスとしてパッケージされたものを含む）も含む。

【０１０８】

ここに記載の検出方法には、その他の組成物が利用され、また有用である。これら組成物に関して、アデノウイルスベクターは通常、緩衝液システムの様な適当な溶媒、又は溶液に懸濁されている。この様な溶媒は当分野で公知である。

本発明のキット

本発明は本発明のアデノウイルスベクターを含むキットも包含する。これらキットは診断及び／又はモニタリングの目的、好ましくはモニタリングに使用できる。これらキットの使用方法は、臨床検査施設、実験研究室、医療現場、又は私的個人によって実施できる。本発明に包含されるキットは、例えば生検標本の様な好適な生物学的サンプル内にある細胞状態産生細胞の存在の検出を可能にする。

【０１０９】

本発明のキットは、好適パッケージ内にここに記載したアデノウイルスベクターを含んでいる。キットは随意緩衝液、現像液、標識体、反応表面、検出手段、コントロールサンプル、装置及び解釈に関する情報を含む操作に於いて有用な追加の成分も提供できるが、もとよりこれに限定されない。

本発明のアデノウイルスベクターの調製

本発明のアデノウイルスベクターは、当分野で標準の組換え体技術により調製できる。一般に細胞状態特異的ＴＲＥは所望のアデノウイルス遺伝子、１又はそれ以上の初期遺伝子の５'側に挿入される（後期遺伝子も利用できるが）。細胞状態特異的ＴＲＥはオリゴヌクレオチド合成（配列が既知の場合）又は組換え体

法（ＰＣＲ及び／又は制限酵素法）を用い調製できる。天然のアデノＤＮＡ配列内にある、又はオリゴヌクレオチド標的突然変異誘導法やＰＣＲの様な方法により誘導された好都合な制限酵素部位は、細胞状態特異ＴＲＥに適した挿入部位を提供する。従って、細胞状態特異的ＴＲＥのアニーリング（即ち挿入）に適した好都合な制限酵素部位は、ＰＣＲの様な標準的組換え体法を用い、細胞状態特異的ＴＲＥの５’及び３’端に作ることができる。

【０１１０】

本発明のアデノウイルスベクターの作製に利用されるポリヌクレオチドは、化学的合成の様な標準的方法を利用し、組換え体法により、及び／又は生物学的資源より所望の配列を得ることにより得ることができるだろう。

アデノウイルスベクターは１方のプラスミドがアデノウイルスの左方の領域を提供し、もう一方のプラスミドが右手の領域を提供し、それらが相同的組み換えに適した中央部の少なくとも５００ｎｔの領域を共有している２個のプラスミドを利用することで好都合に調製される。この方法では、希望する場合には各プラスミドは独立に加工された後、コンピテント宿主細胞に共トランフクションされ、適当であれば相補遺伝子が提供され、あるいはアデノウイルスの増殖に関係した細胞状態特異的ＴＲＥ由来の転写開始に関する適当な転写因子的が提供される。プラスミドは一般にはカチオン性リポソームの様な適当なトランスダクション法を用い、２９３細胞の様な好適宿主細胞内に導入される。又はアデノウイルスゲノムの左方及び右方部分のインビトロ連結を利用し、複製に必須な全てのアデノウイルスゲノム部分を含む組換え体アデノウイルス誘導体を構築する。Ｂｅｒｋｎｅｒら、（１９８３）「Ｎｕｃｌｅｉｃ Ａｃｉｄ Ｒｅｓｅａｒｃｈ」１１：６００３－６０２０；Ｂｒｉｄｇｅｓ、（１９８９）「Ｈ．Ｖｉｒｏｌ．」６３：６３１－６３８。

【０１１１】

都合の良いことに、アデノウイルスの必要部分を提供するプラスミドが利用できる。プラスミドｐＸＣ．１（ＭｃＫｉｎｎｏｎ（１９８２）「Ｇｅｎｅ」１９：３３－４２）はＡｄ５の野生型左端を含む。ｐＢＨＧ１０（Ｂｅｔｔら（１９９４）「Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ」９１：８８０

２－８８０６；Ｍｉｃｒｏｂｉｘ Ｂｉｏｓｙｓｔｅｍｓ Ｉｎｃ．，Ｔｏｒｏｎｔｏ）はＥ３を欠くＡｄ５右端を提供する。Ｅ３の欠失により内因性エンハンサー／プロモーターを欠失すること無しに３ｋｂの細胞状態ＴＲＥをウイルス内に挿入するための空間が提供される。Bettら、(1994)。Ｅ３の遺伝子はＥ４の反対鎖上に位置している（ｒ－鎖）。ｐＢＨＧ１１はより大きなＥ３欠失を提供する（更に０．３ｋｂが欠失している）。Bettら(1994)。

【０１１２】

初期遺伝子の操作に関し、ＡｄＳＥ１Ａの転写開始部位はウイルスゲノムの４９８に位置し、Ｅ１Ａ蛋白質のＡＴＧ開始部位はウイルスゲノムの５６０に位置している。この領域は細胞状態特異的ＴＲＥの挿入に利用できる。制限酵素部位はＡｄ５ゲノムに限定されて利用されるプライマー、又はＡｄ５ゲノムＤＮＡを持つプラスミドの一部を含むプライマーを利用するポリメラーゼ連鎖反応（ＰＣＲ）を実施することで導入される。例えば、ｐＢＲ３２２を利用する場合、プライマーはｐＢＲ３２２主鎖内のＥｃｏＲＩ部位及びＡｄ５の１３３９位置にあるＸｈａＩ部位を利用するだろう。領域中央部に重複するプライマーによって特異的な制限酵素部位が生まれる様な３０配列変化を導入する２段階のＰＣＲを実施することで、その部位に相同的ＴＲＥの挿入ができる。

【０１１３】

同様の方針はＥ１Ｂを制御する異種ＴＲＥの挿入にも利用できるだろう。Ａｄ５のＥ１Ｂプロモーターは、ＳｐＩ及びＴＡＴＡボックスに対する単一の高親和性認識部位である。この領域は１６３６から１７０１に伸びている。この領域に異種ＴＲＥが挿入されることで、Ｅ１Ｂ遺伝子の様的細胞特異的転写を提供できる。異種ＴＲＥにより左領域を修飾し、Ｅ１Ｂを制御する異種ＴＲＥを導入するための鋳型となるＥ１Ａを得ることで、得られたアデノウイルスベクターはＥ１Ａ及びＥ１Ｂの両方の発現に関し細胞状態特異的転写因子に依存する様になる。

【０１１４】

同様に、細胞状態特異的ＴＲＥはＥ２遺伝子の上流に挿入でき、それによりその発現を細胞状態特異的にできる。２７０５０～２７１５０のＡｄ５内にマッピングされるＥ２初期プロモーターは、主要転写開始部位及びＥ２転写体の約５％

に相当する副次的転写開始部位、２個の非典型的ＴＡＴＡボックス、２カ所のＥ２Ｆ転写因子結合部位及びＡＴＦ転写因子結合部位より構成される。Ｅ２プロモーター構造の詳細についてはＳｗａｍｉｎａｔｈａｎら，「Ｃｕｒｒ．Ｔｏｐｉｃｓ ｉｎ Ｍｉｃｒｏ，ａｎｄ Ｉｍｍ」（１９９５） １９９（ｐａｒｔ ３）：１７７－１９４を参照。

【０１１５】

Ｅ４に関しては，アデノウイルスゲノムの右手部分を使用しなければならない、Ｅ４転写開始部位は３５６０９近傍であり、ＴＡＴＡボックスは３５６３８に、そしてＯＲＦ１の最初のＡＴＧ／ＣＴＧは３５５３２にある。Ｖｉｒｔａｎｅｎら（１９８４）「Ｊ．Ｖｉｒｏｌ．」５１：８２２－８３１，他の遺伝子に関する上記方針の何れかを利用し、細胞状態特異的ＴＲＥは転写開始部位上流に挿入される。Ｅ４領域の変異体の構築に関しては、Ｗ１６２細胞内にて共トランスフェクション及び相同組み換えを実施し、トランスのＥ４蛋白質をこれら蛋白質の合成を欠失している相補欠損体に提供する（Ｗｅｉｎｂｅｒｇら、（１９８３）「Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ Ｓｃｉ．」８０：５３８３－５３８６）。あるいは、これら構築体はインビトロ連結によって作製できる。

【０１１６】

本発明のアデノウイルスベクターを利用する方法

本発明のアデノウイルスベクターは所望する、又は意図する結果が様々である広範囲の目的に使用できる。従って本発明は上記のアデノウイルスベクターの使用に関する方法を含む。

実施態様の一つでは、方法は細胞をここに記載したアデノウイルスベクターに、このアデノウイルスベクターが細胞内に侵入する様に接触させることを含む、一般には個体（好ましくはヒト）内にあるものの必ずしもその必要はない標的細胞（即ち細胞状態特異的ＴＲＥを機能させるのに必要な生理状態を提示する細胞）に選択的細胞傷害性を付与するために提供される。細胞傷害性は、色素排除法、^{3}Ｈ－チミジン取り込み及び／又は溶解法の様な当分野標準的なアッセイ法を用い測定することができる。

【０１１７】

別の実施態様では、方法は細胞状態特異的ＴＲＥを機能可能にする哺乳動物細胞に関し特異的なアデノウイルスを増殖するために提供される。これら方法はアデノウイルスベクターを哺乳動物細胞に組合せ、それによって該アデノウイルスが増殖することを必要とする。

本発明は更に腫瘍細胞をここに記載したアデノウイルスベクターに、このアデノウイルスベクターが腫瘍細胞内に侵入し、腫瘍細胞に選択的細胞傷害性を提示する様に接触させることを含む、一般には個体（好ましくはヒト）内にあるものの必ずしもその必要はない腫瘍細胞の増殖を抑制する方法を提供する。腫瘍細胞の増殖は、腫瘍サイズの測定、^{3}Ｈ－サミジン取り込みアッセイ又は腫瘍細胞数を測定することによる腫瘍細胞の増殖有無の決定を含む、当分野で既知の何れかの方法により評価することができるが、もとより上記方法に限定されるわけではない。

【０１１８】

本発明は更に生物学的サンプル中にある標的細胞（即ち細胞状態特異的ＴＲＥを機能させるのに必要な生理状態を提示する細胞）を検出するための方法を含む。これらの方法は特に、実験的又は臨床状況に於いて、個体（即ち哺乳動物）の臨床及び／又は生理学的状態をモニターするのに有用である。これらの方法では、生物学的サンプルの細胞はアデノウイルスに接触させられ、アデノウイルスベクターの複製が検出される。好適な生物学的サンプルは、その中で必要とされる生理学的（及び／又は環境）状態、例えば異常な生理状態（低酸素状態にある、及びＨＩＦ－１の抑制の様な低酸素状態にある細胞に特徴的な表現形を示す細胞）が存在するか、その存在が疑われるサンプルである。

【０１１９】

一般に、哺乳動物では好適な臨床サンプルは、その中で低酸素状態にある固形癌の中にある細胞の様な、必要とされる生理状態を提示している癌細胞の存在が疑われるサンプルである。この様な細胞は、例えば針生検、又はその他の外科的手法により得ることができる。接触を受ける細胞は、アッセイ条件を促進するために、例えば選択的濃縮及び／又は可溶化の様な処理を受けるだろう。これらの方法では、標的細胞はアデノウイルスの増殖を検出する、当分野で標準的である

インビトロアッセイを使用し検出できる。この様な標準的アッセイの例には、バーストアッセイ（ウイルス産出量を測定する）及びプラークアッセイ（細胞当たりの感染細胞を測定する）が含まれるが、これに限定されない。増殖は、特異的アデノウイルスＤＮＡ複製を測定することによっても検出でき、これも標準的アッセイである。

【０１２０】

本発明を例示するために以下実施例を提供するが、これにより本発明は制限されない。

実施例

実施例１

低酸素反応性エレメント及びＰＳＡ－ＴＲＥの転写制御下にあるＥ１Ａを含むアデノウイルスベクター

一般技術

ヒト胚性腎臓細胞株、２９３は効率的にＡｄ５のＥ１Ａ及びＥ１Ｂ遺伝子を発現し、アデノウイルスＤＮＡによる高トランスフェクション率を示す。組換え体アデノウイルスを作製するために、２９３細胞を１種類の左端Ａｄ５プラスミドと１種類の右端Ａｄ５プラスミドで共トランスフェクションした。相同組み換えにより、Ｅ１Ａ及びＥ１Ｂ蛋白質をトランスに提供し、それら蛋白質の合成の欠損を補う、２９３細胞内での増殖に必須な遺伝子エレメントを持つアデノウイルスが作製された。

【０１２１】

組み合わせられるプラスミドはリポフェクチン（ＤＯＴＭＡ：ＤＯＰＥ（商標）、Ｌｉｆｅ　Ｔｅｃｈｎｏｌｏｇｉｅｓ）の様なカチオン性リポソームを使用し、２種類のプラスミドを組合せ、次にプラスミドＤＮＡ液（血清及びその他の添加物を含まない最少必須培地（ＭＥＭ）５００μｌ中に各プラスミドを１０μｌ含む）を４倍モル過剰濃度のリポソームを含む２００μｌの同一緩衝液と混合することで２９３細胞内に共トランスフェクトされた。次にＤＮＡ－液体複合体を細胞上に置き、３７℃、５％CO_2で１６時間インキュベーションした。インキュベーション後、培地を１０％胎児ウシ血清を含むＭＥＭと交換し、細胞を更

に３７℃、５％CO_2で１０日間、培地を２回交換しながらインキュベーションした。終了時に、細胞及び培地をチューブに移し、３回凍結溶解し、溶解物をプラークとして個々のウイルスを検出するのに適当な希釈率で使い２９３細胞に感染させた。

【０１２２】

得られたプラークについて２回プラーク精製し、ＰＣＲ及び必要に応じＤＮＡ配列決定を行い、所望する配列の存在についてウイルスを特徴付けた。その後の実験の為に、ウイルスは塩化セシウム勾配遠心分離法により大規模で精製された。

ＥＩＡが細胞状態特異的ＴＲＥの転写制御下にあるアデノウイルスベクター

低酸素反応エレメント（ＨＲＥ）を含むアデノウイルスベクターが作製された。ＥＩＡがＨＲＥ及びＰＳＡ−ＴＲＥより成る複合ＴＲＥの制御下にあるアデノウイルスベクターであるＣＮ７９６は、ＣＮ５１５をｐＢＨＧ１０と共トランスフェクションすることで作製された。ＣＮ５１５はＨＥＲｅｎｏ１（Ｊｉａｎｇら、（１９９７）「Ｃａｎｃｅｒ　Ｒｅｓｅａｃｈ」５７：５３２８−５３３５）（配列番号１：図２）由来の６７塩基対断片を、ＣＮ６５のＢｇＩＩＩ部位に挿入することで構築された。ＣＮ６５は、−５４１から＋１２間でのＰＳＡプロモーターに融合した−５３２２から−３７３８までのエンハンサーから成るヒトＰＳＡ由来のエンハンサー及びプロモーターを含むプラスミドである。これは、プラスミド７０６内に含有されたＰＳＡ−ＴＲＥである。Ｒｏｄｒｉｇｕｅｚら「Ｃａｎｃｅｒ　Ｒｅｓ．」５７：２５５９−２５６３。

【０１２３】

インビトロでのウイルス増殖

組換え体アデノウイルスの増殖選択性はプラークアッセイにて分析し、その中で感染と複製を繰り返し、単一感染粒子は視認できるプラークを生じた。保存ウイルスを等しいｐｆｕ／ｍｌに希釈し、続いて細胞の単層に１時間感染した。接種物を取り出し、培地を含む半固形寒天を細胞に重層し、３７℃で１０日間インキュベーションした。次に単層内のプラークを数え、各種細胞に於ける感染ウイルスの力価を計算した。データは２９３細胞に対するＣＮ７０２（野生型）の力

値で標準化した。

【図面の簡単な説明】

【図１】

図１は実施例１記載の如くＥ１Ａ遺伝子がＨＲＥ及びＰＳＡ－ＴＲＥの転写制御下にあるアデノウイルスベクターＣＮ７９６の概略図である。

【図２】

図２は、ラットエノラーゼ－１遺伝子の５’フランキング領域にあるＨＲＥのヌクレオチド配列（配列番号１）を示す。

【図３】

図３は、ヒトＥ２Ｆ１遺伝子の５’フランキング領域のヌクレオチド配列（配列番号２）を示す。星印は転写開始部位を示す。

【図４】

図４は前立腺特異的抗原ＴＲＥのヌクレオチド配列を示す。

【図５】

図５は癌胚抗原ＴＲＥのヌクレオチド配列を示す。

【図６】

図６はヒト腺型カリクレインＴＲＥのヌクレオチド配列を示す。

【図７】

図７はムチンＴＲＥのヌクレオチド配列を示す。

【図８】

図８はラットプロバシンＴＲＥのヌクレオチド配列を示す。

【図９】

図９はアデノウイルス死滅蛋白質のヌクレオチド配列及び翻訳されたアミノ酸配列を示す。

【図1】

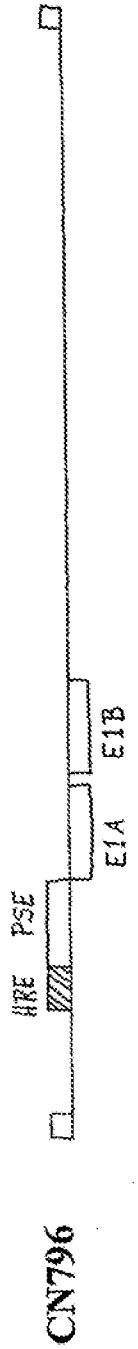


FIGURE 1

【図2】

FIGURE 2

ccccgagg cagtgcat gaggctcagg gcgtgcgt gagtcgcagcgagaccccg gggtgcag gccgga

【図3】

FIGURE 3

```
gggcccaaaa ttagcaagtg accacgtggt tctgaagcca gtggcctaag gaccaccctt 61
gcagaaccgt ggtctccttg tcacagtcta ggcagcctct ggcttagcct ctgtttcttt 121
cataaccttt ctcagcgcct gctctgggcc agaccagtgt tgggaggagt cgctactgag 181
ctcctagatt ggcaggggag gcagatggag aaaaggagtg tgtgtggtca gcattggagc 241
agaggcagca gtgggcaata gaggaagtga gtaaatcctt gggagggctc cctagaagtg 301
atgtgttttc tttttttgtt ttagagacag gatctcgctc tgtcgcccag gctggtgtgc 361
agtggcatga tcatagctca ctgcagcctc gacttctcgg gctcaagcaa tcctcccacc 421
tcagcctccc aagtagctgg gactacgggc acacgccacc atgcctggct aatttttgta 481
tttttgtag agatgggtct tcaccatgtt gatcaggctg gtctcgaact cctgggctca 541
tgcgatccac ccgccagct gattacaggg attccggtgg tgagccaccg cgcccagacg 601
ccacttcatc gtattgtaaa cgtctgttac ctttctgttc ccctgtctac tggactgtga 661
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ctaaaatggg acttgcagga aagcccgacg tccccctcgc catttccagg caccgctctt 841
cagcttgggc tctgggtgag cgggataggg ctgggtgcag gattaggata atgtcatggg 901
tgaggcaagt tgaggatgga agaggtggct gatggctggg ctgtggaact gatgatcctg 961
aaaagaagag gggacagtct ctggaaatct aagctgaggc tgttgggggc tacaggttga 1021
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ggcagccaat tgtggcggcg ctcggcggct cgtggctctt tcgcggcaaa aaggatttgg 1381
cgcgtaaaag*tggccgggac tttgcaggca gcggcggccg gggcggagc gggatcgagc 1441
cctcgccgag gcctgccgcc atgggcccgc gccgccgccg ccgcctgtca cccgggccgc 1501
gcgggccgtg agcgtcatg
```

【図４Ａ】

FIGURE 4A

aagcttctag tttttttttc ccggtgacat cgtggaaagc actagcatct ctaagcaatg 60

atctgtgaca atattcacag tgtaatgcca tccagggaac tcaactgagc cttgatgtcc 120

agagatttttt gtgtttttttt ctgagactga gtctcgctct gtgcccaggct ggagtgcagt 180

ggtgcaacct tggctcactg caagctccgc ctcctgggtt cacgccattc tcctgcctca 240

gcctcctgag tagctgggac tacaggcacc cgccaccacg cctggctaat ttttttgtat 300

ttttagtaga gatggggttt cactgtgtta gccaggatgg tctcagtctc ctgacctcgt 360

gatctgccca ccttggcctc ccaaagtgct gggattgacag gcgtgagcca ccgcgcctgg 420

ccgatatcca gagattttttt gggggctcc atcacacaga catgttgact gtcttcatgg 480

ttgacttttta gtatccagcc cctctagaaa tctagctgat atagtgtggc tcaaaacctt 540

cagcacaaat cacaccgtta gactatctgg tgtggccaa accttcaggc gaacaaaggg 600

actctaatct ggcaggatat tccaaagcat tagagatgac ctcttgcaaa gaaaaagaaa 660

tggaaaagaa aaagaaagaa aggaaaaaaa aaaaaaaaaa gagatgacct ctcaggctct 720

gagggaaaac gcctgaggtc tttgagcaag gtcagtcctc tgttgcacag tctcccctcac 780

agggtcattg tgacgatcaa atgtggtcac gtgtatgagg caccagcaca tgcctggctc 840

tggggagtgc cgtgtaagtg tatgcttgca ctgctgaatg cttgggatgt gtcagggatt 900

atcttcagca cttacagatg ctcatctcat cctcacagca tcactatggg atgggtatta 960

ctggcctcat ttgatggaga aagtggctgt ggctcagaaa ggggggacca ctagaccagg 1020

gacactctgg atgctgggga ctccagagac catgaccact caccaactgc agagaaatta 1080

attgtggcct gatgtccctg tcctggagag ggtggaggtg gaccttcact aacctcctac 1140

cttgacccta tcttttaggg ctctttctga cctccaccat ggtactagga ccccattgta 1200

ttctgtaccc tcttgactct atgccccca ctgcccactg catccagctg ggtccccctcc 1260

tatctctatt cccagctggc cagtgcagtc tcagtgccca cctgtttgtc agtaactctg 1320

aagggctga cattttactg acttgcaaac aaataagcta actttccaga gttttgtgaa 1380

tgctggcaga gtccatgaga ctcctgagtc agaggcaaag gcttttactg ctcacagctt 1440

agcagacagc atgaggttca tgttcacatt agtacacctt gccccccccca aatcttgtag 1500

ggtgaccaga gcagtctagg tggatgctgt gcagaagggg tttgtgccac tggtgagaaa 1560

cctgagatta ggaatcctca atcttatact gggacaactt gcaaacctgc tcagcctttg 1620

tctctgatga agatattatc ttcatgatct tggattgaaa acagacctac tctggaggaa 1680

【図４Ｂ】

FIGURE 4B

catattgtat cgattgtcct tgacagtaaa caaatctgtt gtaagagaca ttatctttat 1740
tatctaggac agtaagcaag cctggatctg agagagatat catcttgcaa ggatgcctgc 1800
tttacaaaca tccttgaaac aacaatccag aaaaaaaaag gtgttgctgt ctttgctcag 1860
aagacacaca gatacgtgac agaaccatgg agaattgcct cccaacgctg ttcagccaga 1920
gccttccacc cttgtctgca ggacagtctc aacgttccac cattaaatac ttcttctatc 1980
acatcctgct tctttatgcc taaccaaggt tctaggtccc gatcgactgt gtctggcagc 2040
actccactgc caaacccaga ataaggcagc gctcaggatc ccgaaggggc atggctgggg 2100
atcagaactt ctgggtttga gtgaggagtg ggtccaccct cttgaatttc aaaggaggaa 2160
gaggctggat gtgaaggtac tgggggaggg aaagtgtcag ttccgaactc ttaggtcaat 2220
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ttccatcttg ccacctaatc cttactccac ttgagggtat caccagccct tctagctcca 2520
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gctgtgacta tcctgcagc gtgcctctcc agccacctgc caaccgtaga gctgcccatc 2700
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ggatgagttt gggattgaca ctgtggaggt ggtttgggat ggcatggctt tgggatggaa 3360

【図４Ｃ】

FIGURE 4C

stagatttgt tttgatgttg gctcagacat ccttggggat tgaactgggg atgaagctgg 3420

gtttgatttt ggaggtagaa gacgtggaag tagctgtcag actttgacagt ggccatgagt 3480

tttgtttgat ggggaatcaa acaatgggag aagacataag ggttggcttg ttaggttaag 3540

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catgaggttc tcactggagt ggagacaaac ttcctttcca ggatgaatcc agggaagcct 3720

taattcacgt gtaggggagg tcaggccact ggctaagtat atccttccac tccagctcta 3780

agatggtctt aaattgtgat tatctatatc cacttctgtc tcgctcactg tgcttggagt 3840

ttacctgatc actcaactag aaacagggga agattttatc aaatttcttt ttttttttt 3900

ttttttttga gacagagtct cactctgttg cccaggctgg agtgcagtgg cgcagtctcg 3960

gctcactgca acctctgcct cccaggttca agtgattctc ctgcctcagc ctcctgagtt 4020

gctgggatta caggcatgca gcaccatgcc cagctaattt ttgtattttt agtagagatg 4080

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cagcctccca aagtgctggg attacaggcg tcagccaccg cgcccagccc cttttgtcaa 4200

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aaataaccaa ctttttggaa attgatgaaa tcttacggag ttaacagtgg aggtaccagg 4320

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tgcttcggtc tcccaatagc taagactaca gtagtcccacc accatatccc gataatttct 4560

aaatttttg ggggcccggg cacagtggct cacgcctgta atcccaacac catgggaggc 4620

tgagatgggt ggatcacgag gtcaggagtt tgagaccagc ctgaccaaca tggtgaaact 4680

ctgtctctac taaaaaaaaa aaaaatagaa aaattagccg ggcgtggtgg cacacggcac 4740

ctgtaatccc agctactgag gaggctgagg caggagaatc acttgaaccc agaaggcaga 4800

ggttgcaatg agccgagatt gcgccactgc actccagcct gggtgacaga gtgagactct 4860

gtctcaaaaa aaaaaaattt ttttttttt tttgtagaga tggatcttgc tttgtttctc 4920

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ggattacagg cgtgagccac catgactgac ctgtcgttaa tcttgaggta cataaacctg 5040

gctcctaaag gctaaaggct aaatatttgt tggagaaggg gcattggatt ttgcatgagg 5100

【図4D】

FIGURE 4D

atgattctga cctgggaggg caggtcagca ggcatctctg ttgcacagat agagtgtaca 5160
ggtctggaga acaaggagtg gggggttatt ggaattccac attgtttgct gcacgttgga 5220
ttttgaaatg ctagggaact ttgggagact catatttctg ggctagagga tctgtggacc 5280
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gctggtgtct tagggcacac tgggtcttgg agtgcaaagg atctaggcac gtgaggcttt 5520
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tgggaggggg ttgtccagcc tccagcagca tggggagggc cttggtcagc ctctgggtgc 5760
cagcagggca ggggcggagt cctggggaat gaaggtttta tagggctcct gggggaggct 5820
ccccagcccc aagctt 5836

【図5A】

FIGURE 5A

aagctttta gtgctttaga cagtgagctg gtctgtctaa cccaagtgac ctgggctcca 60

tactcagccc cagaagtgaa gggtgaagct gggtggagcc aaaccaggca agcctaccct 120

cagggctccc agtggcctga gaaccattgg acccaggacc cattacttct agggtaagga 180

aggtacaaac accagatcca accatggtct gggggacag ctgtcaaatg cctaaaaata 240

tacctgggag aggagcaggc aaactatcac tgccccaggt tctctgaaca gaaacagagg 300

ggcaacccaa agtccaaatc caggtgagca ggtgcaccaa atgcccagag atatgacgag 360

gcaagaagtg aaggaaccac ccctgcatca aatgttttgc atgggaagga gaagggggtt 420

gctcatgttc ccaatccagg agaatgcatt tgggatctgc cttcttctca ctccttggtt 480

agcaagacta agcaaccagg actctggatt tggggaaaga cgtttatttg tggaggccag 540

tgatgacaat cccacgaggg cctaggtgaa gaggggcagga aggctcgaga cactggggac 600

tgagtgaaaa ccacacccat gatctgcacc acccatggat gctccttcat tgctcaactt 660

tctgttgata tcagatggcc ccatttttcg tacattcaca gaaggacaca ggctagggtc 720

tgtgcatggc attcatcccc ggggccatgt gaggacagca ggtgggaaag atcatgggtc 780

ctcctgggtc ctgcagggcc agaacattca tcacccatac tgacctccta gatgggaatg 840

gcttccctgg ggctgggcca acggggcctg ggcaggggag aaaggacgtc aggggacagg 900

gaggaagggt catcgagacc cagcctggaa ggttcttgtc tctgaccatc caggatttac 960

ttcccctgcat ctaccctttgg tcattttccc tcagcaatga ccagctctgc ttcctgatct 1020

cagcctccca ccctggacac agcacccccag tccctggccc ggctgcatcc acccaatacc 1080

ctgataaccc aggacccatt acttctaggg taaggagggt ccaggagaca gaagctgagg 1140

aaaggtctga agaagtcaca tctgtcctgg ccagagggga aaaaccatca gatgctgaac 1200

caggagaatg ttgacccagg aaagggaccg aggacccaag aaaggagtca gaccaccagg 1260

gtttgcctga gaggaaggat caaggccccg agggaaagca gggctggctg catgtgcagg 1320

acactggtgg ggcatatgtg tcttagattc tccctgaatt cagtgtccct gccatggcca 1380

gactcctctac tcaggcctgg acatgctgaa ataggacaat ggccttgtcc tctctccccc 1440

ccatttggca agagacataa aggacattcc aggacatgcc ttcctgggag gtccaggttc 1500

tctgtctcac acctcaggga ctgtagttac tgcatcagcc atggtaggtg ctgatctcac 1560

ccagcctgtc caggcccttc cactctccac tttgtgacca tgtccaggac cacccctcag 1620

atcctgagcc tgcaaatacc cccttgctgg gtgggtggat tcagtaaaca gtgagctcct 1680

【図5B】

FIGURE 5B

atccagcccc cagagccacc tctgtcacct tcctgctggg catcatccca ccttcacaag 1740
cactaaagag catggggaga cctggctagc tgggtttctg catcacaaag aaaatcatcc 1800
cccaggttcg gattcccagg gctctgtatg tggagctgac agacctgagg ccagggagata 1860
gcagaggtcc gccctaggga gggtgggtca tccacccagg ggacaggggt gcaccagcct 1920
tgctactgaa agggcctccc caggacagcg ccatcagccc tgcctgagag ctttgctaaa 1980
cagcagtcag aggaggccat ggcagtggct gagctcctgc tccaggcccc aacagaccag 2040
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gcccagatgg ggagatggat gtcaggaaag gctgcccccat cagggagggt gatagcaatg 3060
gggggtctgt gggagtgggc acgtgggatt cctgggctc tgccaagttc cctcccatag 3120
tcacaacctg gggacactgc ccatgaaggg gcgcctttgc ccagccagat gctgctggtt 3180
ctgcccatcc actacccctct ctgctccagc cactctgggt ctttctccag atgcccctgga 3240
cagccctggc ctgggcctgt cccctgagag gtgttgggag aagctgagtc tctggggaca 3300
ctctcatcag agtctgaaag gcacatcagg aaacatcccc ggtctccagg actaggcaat 3360

【図5C】

FIGURE 5C

gaggaaaggg ccccagctcc tccctttgcc actgagaggg tcgaccctgg gtggccacag 3420

tgacttctgc gtctgtccca gtcaccctga aaccacaaca aaaccccagc cccagaccct 3480

gcaggtacaa tacatgtggg gacagtctgt acccaggggа agccagttct ctcttcctag 3540

gagaccgggc ctcagggctg tgccccgggc aggcgggggc agcacgtgcc tgtccttgag 3600

aactcgggac cttaagggtc tctgctctgt gaggcacagc aaggatcctt ctgtccagag 3660

atgaaagcag ctcctgcccc tcctctgacc tcttcctcct tcccaaatct caaccaacaa 3720

ataggtgttt caaatctcat catcaaatct tcatccatcc acatgagaaa gcttaaaacc 3780

caatggattg acaacatcaa gagttggaac aagtggacat ggagatgtta cttgtggaaa 3840

tttagatgtg ttcagctatc gggcaggagc atctgtgtca aattccagca tggttcagaa 3900

gaatcaaaaa gtgtcacagt ccaaatgtgc aacagtgcag gggataaaac tgtggtgcat 3960

tcaaactgag ggatattttg gaacatgaga aaggaagggа ttgctgctgc acagaacatg 4020

gatgatctca cacatagagt tgaaagaaag gagtcaatcg cagaatagaa aatgatcact 4080

aattccacct ctataaagtt tccaagagga aaacccaatt ctgctgctag agatcagaat 4140

ggaggtgacc tgtgccttgc aatggctgtg agggtcacgg gagtgtcact tagtgcaggc 4200

aatgtgccgt atcttaatct gggcagggct ttcatgagca cataggaatg cagacattac 4260

tgctgtgttc attttacttc accggaaaag aagaataaaa tcagccgggc gcggtggctc 4320

acgcctgtaa tcccagcact ttagaaggct gaggtgggca gattacttga ggtcaggagt 4380

tcaagaccac cctggccaat atggtgaaac cccggtctca ctaaaaatac aaaaattagc 4440

tgggcatggt ggtgcgcgcc tgtaatccca gctactcggg aggctgaggc tggacaattg 4500

cttggaccca ggaagcagag gttgcagtga gccaagattg tgccactgca ctccagcttg 4560

ggcaacagag ccagactctg taaaaaaaaa aaaaaaaaaa aaaaaaagaa agaaagaaaa 4620

agaaaagaaa gtataaaatc tctttgggtt aacaaaaaaa gatccacaaa acaaacacca 4680

gctcttatca aacttacaca actctgccag agaacaggaa acacaaatac tcattaactc 4740

actttgtgg caataaaacc ttcatgtcaa aaggagacca ggacacaatg aggaagtaaa 4800

actgcaggcc ctacttgggt gcagaggagg aaaatccaca aatataaacat taccagaagg 4860

agctaagatt tactgcattg agttcattcc ccaggtatgc aaggtgattt taacacctga 4920

aaatcaatca ttgcctttac tacatagaca gattagctag aaaaaaatta caactagcag 4980

aacagaagca atttggcctt cctaaaattc cacatcatat catcatgatg gagacagtgc 5040

agacgccaat gacaataaaa agagggacct ccgtcaccag gtaaacatgt ccacacagct 5100

【図５Ｄ】

FIGURE 5D

ccagcaagca cccgtcttcc cagtgaatca ctgtaacctc cccttcaatc agccccaggc 5160

aaggctgcct gcgatggcca cacaggctcc aacccgtggg cctcaacctc ccgcagaggc 5220

tctcctttgg ccaccccatg gggagagcat gaggacaggg cagagccctc tgatgcccac 5280

acatggcagg agctgacgcc agagccatgg gggctggaga gcagagctgc tggggtcaga 5340

gcttcctgag gacacccagg cctaagggaa ggcagctccc tggatggggg caaccaggct 5400

ccgggctcca acctcagagc ccgcatggga ggagccagca ctctaggcct ttcctagggt 5460

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gaccaagccc aggaccaatg tggaaggaag gaaacagcat cccctttagt gatggaaccc 5700

aaggtcagtg caaagagagg ccatgagcag ttaggaaggg tggtccaacc tacagcacaa 5760

accatcatct atcataagta gaagcctgc tcnatgaccc ctgcatttaa ataaacgttt 5820

gttaaatgag tcaaattccc tcaccatgag agctcacctg tgtgtaggcc catcacacac 5880

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ggaggggcct gcagggagct gggtactgcc ctccagggag ggggctgcag ggagctgggt 6180

actgccctcc agggaggggg ctgcagggag ctgggtactg ccctccaggg agggggctgc 6240

agggagctgg gtactgccct ccagggaggc aggagcactg ttcccaacag agagcacatc 6300

ttcctgcagc agctgcacag acacaggagc ccccatgact gccctgggcc agggtgtgga 6360

ttccaaattt cgtgccccat tgggtgggac ggaggttgac cgtgacatcc aaggggcatc 6420

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ggggtcctcc catgccccca gtctgacttg caggtgcaca gggtggctga catctgtcct 6600

tgctccttct cttggctcaa ctgccgcccc tcctgggggt gactgatggt caggacaagg 6660

gatcctagag ctggccccat gattgacagg aaggcaggac ttggcctcca ttctgaagac 6720

tagggggtgtc aagagagctg ggcatcccac agagctgcac aagatgacgc ggacagaggg 6780

【図５Ｅ】

FIGURE 5E

tgacacaggg ctcagggctt cagacgggtc gggaggctca gctgagagtt cagggacaga 6840

cctgaggagc ctcagtgggg aaagaagcac tgaagtggga agttctggaa tgttctggac 6900

aagcctgagt gctctaagga aatgctccca cccegatgta gcctgcagca ctggacggtc 6960

tgtgtacctc cccgctgccc atcctctcac agccccegcc tctagggaca caactcctgc 7020

cctaacatgc atctttcctg tctcattcca cacaaaaggg cctctggggt ccctgttctg 7080

cattgcaagg agtggaggtc acgttcccac agaccaccca gcaacagggt cctatggagg 7140

tgcggtcagg aggatcacac gtccccccat gcccagggga ctgactctgg gggtgatgga 7200

ttggcctgga ggccactggt cccctctgtc cctgaggggа atctgcaccc tggaggctgc 7260

cacatcgctc ctgattcttt cagctgaggg cccttcttga aatcccaggg aggactcaac 7320

ccccactggg aaaggcccag tgtggacggt tccacagcag cccagctaag gcccttggac 7380

acagatcctg agtgagagaa cctttaggga cacaggtgca cggccatgtc cccagtgccc 7440

acacagagca ggggcatctg gaccctgagt gtgtagctcc cgcgactgaa cccagccctt 7500

ccccaatgac gtgacccctg gggtggctcc aggtctccag tccatgccac caaaatctcc 7560

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aagggaggac atgagctccа ggaagggcga tggcgtcctc tagtgggcgc ctcctgttaa 7740

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tggagaggac tgaggtgcaa agaggggggt gaagtagggg agtggtcggg agagatggga 7860

ggagcaggta aggggaagcc ccagggaggc cgggggaggg tacagcagag ctctccactc 7920

ctcagcattg acatttgggg tggtcgtgct agtggggttc tgtaagttgt agggtgttca 7980

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accacaactg gaatgacact cactgggtta cattacaaag tggaatgtgg ggaattctgt 8400

agactttggg aagggaaatg tatgacgtga gcccacagcc taaggcagtg gacagtccac 8460

tttgaggctc tcaccatcta ggagacatct cagccatgaa catagccaca tctgtcatta 8520

【図５Ｆ】

FIGURE 5F

gaaaacatgt tttattaaga ggaaaaatct aggctagaag tgctttatgc tctttttct 8580
ctttatgttc aaattcatat actttagat cattccttaa agaagaatct atccccctaa 8640
gtaaatgtta tcactgactg gatagtgttg gtgtctcact cccaacccct gtgtggtgac 8700
agtgccctgc ttccccagcc ctgggccctc tctgattcct gagagctttg ggtgctcctt 8760
cattaggagg aagagaggaa gggtgttttt aatatttctca ccattcaccc atcccacctct 8820
tagacactgg gaagaatcag ttgcccactc ttggatttga tcctcgaatt aatgacctct 8880
atttctgtcc cttgtcccatt tcaacaatgt gacagggccta agcggtgcct tctccatgtg 8940
atttttgagg agaaggttct caagataagt tttctcacac ctctttgaat taccttccacc 9000
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ccacctcttg agggtgtata ctgtatgctc tctcacacagg aatatgcaga ggaaatagaa 9120
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taaaatccta agagagccca tacaaaatta ttaccagtgc taaaactaca aaagttacac 9240
taacagtaaa ctagaataat aaaacatgca tcacagttgc tggtaaagct aaatcagata 9300
ttttttcttt agaaaaagca ttccatgtgt gttgcagtga tgacaggagt gccottcagt 9360
caatatgctg cctgtaattt ttgttcccctg gcagaatgta ttgtctttta tccctttaaa 9420
tcttaaatgc aaaactaaag gcagctcctg ggccccctcc ccaaagtcag ctgcctgcaa 9480
ccagccccac gaagagcaga ggcctgagct tccctggtca aaataggggg ctagggagct 9540
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agaccctgc ctggggtcta caacacatat ggactgtgag caccaagtcc agccctgaat 9840
ctgtgaccac ctgccaagat gcccctaact gggatccacc aatcactgca catggcaggc 9900
agcgaggctt ggaggtgctt cgccacaagg cagccccaat ttgctgggag tttcttggca 9960
cctggtagtg gtgaggagcc ttgggaccct caggattact ccccttaagc atagtggggg 10020
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agaccccctgc accaatgaga ccatgctgaa gcctcagaga gagagatgga gctttgacca 10140
ggagccgctc ttccttgagg gccagggcag ggaaagcagg aggcagcacc aggagtggga 10200

【図5G】

FIGURE 5G

acaccagtgt ctaagccccc gatgagaaca gggtggtctc tcccatatgc ccataccagg 10260
cctgtgaaca gaatcctcct tctgcagtga caatgtctga gaggacgaca tgtttcccag 10320
cctaacgtgc agccatgccc atctacccac tgcctactgc aggacagcac caacccagga 10380
gctgggaagc tgggagaaga catggaatac ccatggcttc tcaccttcct ccagtccagt 10440
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cctaggtgcc tctgggaggc cgaggcagga gaattgcttg aacccgggag gcagaggttg 10560
cagtgagccg agatcacacc actgcactcc agcctgggtg acagaatgag actctgtctc 10620
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aaatatagtg gtgaggatgt agaatattgt gaatataatt aacggcattt aattgtacac 10800
ttaacatgat taatgtggca tattttatct tatgtatttg actacatcca agaaacactg 10860
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gggggcacaa acctcagcac tgccaggaca cacaccattc tcgtggattc tgactttatc 11100
tgacccaggcc cactgtccag atcttgttgt gggattggga caagggaggt cataaagcct 11160
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gacacacaga gtgtgtgggtg ccgtgccccc agagtcgggc tctcctgttc tggtccccag 11880
ggagtgagaa gtgaggttga cttgtccctg ctcctctctg ctaccccaac attcaccttc 11940

【図５Ｈ】

FIGURE 5H

tcctcatgcc cctctctctc aaatatgatt tggatctatg tcccgcccca aatctcatgt 12000
caaattgtaa accccaatgt tggaggtggg gccttgtgag aagtgattgg atgatgcggg 12060
tggatttttct gctttgatgc tgtttctgtg atagagctct cacatgatct ggttgtttaa 12120
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atcttctact cccagatccc cgcacacgct tagccccaga catcactgcc cctgggagca 12420
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ctcaccgagc tgaaacctgg tagcactttg gcataacatg tgcatgaccc gtgttcaatg 12600
tctagagatc agtgttgagt aaaacagcct ggtctggggc cgctgctgtc cccacttccc 12660
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cctcttgccc tccaggggct gacattgcac acagacatca ctcaggaaac ggattcccct 13560
ggacaggaac ctggctttgc taaggaagtg gaggtggagc atggtttcca tccctgctca 13620

【図５１】

FIGURE 5I

caacagaccc ttctgatctc tcccacatac ctgctctgtt cctttctggg tcctatgagg 13680

accctgttct gccaggggtc cctgtgcaac tccagactcc ctcctggtac caccatgggg 13740

aaggtggggt gatccacagga cagtcagcct cgcagagaca gagaccaccc aggactgtca 13800

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acctctgtca ccttcctgtt gggcatcatc ccaccttccc agagccctgg agagcatggg 14160

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ggatgggggt ccatccacct tgccgaaaag atttgtctga ggaaactgaaa atagaaggga 14340

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aaagaccaca cccatgaccc acgtgatgct gagaagtact cctgccctag gaagagactc 14460

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tggaactcaa gctcttctcc acagaggagg acagagcaga cagcagagac catggagtct 14580

ccctcggccc ctccccacag atggtgcatc ccctggcaga ggctcctgct cacaggtgaa 14640

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gaactcatcc acaggaatct gcagcctgtc ccaggcactg gggtgcaacc aagatc 15056

【図6A】

FIGURE 6A

gaattcagaa atagggggaag gttgaggaag gacactgaac tcaaagggga tacagtgatt 60

ggtttatttg tattctcttc acaacattgg tgctggagga attcccaccc tgaggttatg 120

aagatgtctg aacacccaac acatagcact ggagatatga gctcgacaag agttttctag 180

ccacagagat tcacagccta gggcaggagg acactgtacg ccaggcagaa tgacatggga 240

attgcgctca cgattggctt gaagaagcaa ggactgtggg aggtgggctt tgtagtaaca 300

agagggcagg gtgaactctg attcccatgg gggaatgtga tggtcctgtt acaaattttc 360

caagctggca gggaataaaa cccattacgg tgaggacctg tggagggcgg ctgccccaac 420

tgataaagga aatagcccgg tgggggcctt tcccattgta gggggacat atctggcaat 480

agaagccttt gagacccttt agggtacaag tactgaggca gcaaataaaa tgaaatctta 540

tttttcaact ttatactgca tgggtgtgaa gatatatttg tttctgtacc gggggtgagg 600

gaaaggaggg gaggaggaaa gttcctgcag gtctggtttg gtcttgtgat ccaggggctc 660

ttggaactat ttaaattaaa ttaaattaaa acaagcgact gttttaaatt aaattaaatt 720

aaattaaatt ttactttatt ttatcttaag ttctgggcta catgtgcagg acgtgcagct 780

ttgttacata ggtaaacgtg tgccatggtg gtttgctgta cctatcaacc catcacctag 840

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aaaatatgca tgagtagcag aatgtaaaag aggtgaagaa cgtaatagtg actttttaga 1320

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gtgaataaat agtagagaca tgtttgatgg atttttaaaat atttgaaaga cctcacatca 1500

aaggattcat accgtgccat tgaagaggaa gatggaaaag ccaagaagcc agatgaaagt 1560

tagaaatatt attggcaaag cttaaatgtt aaaagtccta gagagaaagg atggcagaaa 1620

tattggcggg aaagaatgca gaacctagaa tataaattca tcccaacagt ttggtagtgt 1680

【図6B】

FIGURE 6B

gcagctgtag cctttttctag atgatacact attgtcatac atcgcttaag cgagtgtaaa 1740

atggtctcct cactttattt atttatatat ttatttagtt ttgagatgga gcctcgctct 1800

gtctcctagg ctggagtgca atagtgcgat accactcact gcaacctctg cctcctctgt 1860

tcaagtgatt ttcttacctc agcctcccga gtagctggga ttacaggtgc gtgccaccac 1920

acccggctaa tttttgtatt tttgtagag acggggtttt gccatgttgg ccaggctggt 1980

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ggccatgcag tattccatat tgcgtataga tcacattttc tttctttttt ttttttgaga 2460

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caaagtgctg ggattacagg ggtgagccac tgcgcccggc ccatatatac cacatttttt 2760

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aacagtatat aagcatttcc ttttctccac agctttgtca tcatggtttt ttttttcttt 3060

tattttaaaa aagaatatgt tgttgttttc ccagggtaca tgtgcaggat gtgcaggttt 3120

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ggtatgaagc cctgcctgca ttagctcttt tccctaatgc tctcactact gcccaccct 3240

cacccctgaca gggcaaacag acaacctaca gaatgggagg aaatttttgc aatctattca 3300

tctgacaaag gtcaagaata tccagaatct acaaggaact taagcaaatt tttacttttt 3360

【図6C】

FIGURE 6C

aataatagcc actctgactg gcgtgaaatg gtatctcatt gtggttttca tttgaatttc 3420
tctgatgatc agtgacgatg agcatttttt catatttgtt ggctgcttgt acgtcttttg 3480
agaagtgtct cttcatgcct tttggccact ttaatgggat tatttttgc cttttagttt 3540
aagttcctta tagattctgg atattagact tcttattgga tgcatagttt gtgaatactc 3600
tcttccattc tgtaggttgt ctgtttactc tattgatggc tttttttgct gtgccgaagc 3660
atcttagttt aattagaaac cacctgccaa tttttgtttt tgttgcaatt gcttttgggg 3720
acttagtcat aaactctttg ccaaggtctg ggtcaagaag agtatttcct aggttttctt 3780
ctagaatttt gaaagtctga atgtaaacat ttgcattttt aatgcatctt gagttagttt 3840
ttgtatatgt gaaaggtcta ctctcatttt ctttcctctt ttctttcttt ctttcttttc 3900
tttctttctt tctttctttc tttctttctt tctttctttc tttctttttg tcttctttc 3960
tttctttctt tctctttctt tctctctttc tttttttttt ttgatggagt attgctctgt 4020
tgcccaggct gcagtgcagc ggcacgatct cggctcactg caacctctgc ctcctgggtt 4080
caactgattc tcctgcatca gcctttccaag tagctgggat tataggcgcc cgccaccacg 4140
cccgactaat ttttgtattt ttagtagaga cggggttgtg ccatgttggc caggctggtt 4200
tgaaactcct gacctcaaac gatctgcctg ccttggcctc ccaaagtgct gggattacag 4260
gtgtgagcca ctgtgcccag ccaagaatgt catttttctaa gaggtccaag aacctcaaga 4320
tattttggga ccttgagaag agaggaattc atacaggtat tacaagcacc gcctaatggc 4380
aaatctttgg catggcttgg cttcaagact ttaggctctt aaaagtcgaa tccaaaaatt 4440
tttataaaag ctccagctaa gctaccttaa aaggggcctg tatggctgat cactctttct 4500
gctatacttt acacaaataa acaggccaaa tataatgagg ccaaaatttа ttttgcaaat 4560
aaattggtcc tgctatgatt tactcttggt aagaacaggg aaaatagaga aaaatttaga 4620
ttgcatctga cctttttttc tgaatttttа tatgtgccta caattttgagc taaatcctga 4680
attatttttct ggttgcaaaa actctctaaa gaagaacttg gttttcattg tcttcgtgac 4740
acatttatct ggctctttac tagaacagct ttcttgtttt tggtgttcta gcttgtgtgc 4800
cttacagttc tactcttcaa attattgtta tgtgtatctc atagttttcc ttcttttgag 4860
aaaactgaag ccatggtatt ctgaggacta gagatgactc aacagagctg gtgaatctcc 4920
tcatatgcaa tccactgggc tcgatctgct tcaaattgct gatgcactgc tgctaaagct 4980
atacatttaa aacccctcact aaaggatcag ggcccatcat ggaagaggag gaaacatgaa 5040
attgtaagag ccagattcgg ggggtagagt gtggaggtca gagcaactcc accttgaata 5100

【図6D】

FIGURE 6D

agaaggtaaa gcaacctatc ctgaaagcta acctgccatg gtggcttctg attaacctct 5160

gttctaggaa gactgacagt ttgggtctgt gtcattgccc aaatctcatg ttaaattgta 5220

atccccagtg ttcggaggtg ggacttggtg gtaggtgatt cggtcatggg agtagatttt 5280

cttctttgtg gtgttacagt gatagtgagt gagttctcgt gagatctggt catttaaaag 5340

tgtgtggccc ctcccctccc tctcttggtc ctcctactgc catgtaagat acctgctcct 5400

gctttgcctt ctaccataag taaaagcccc ctgaggcctc cccagaagca gatgccacca 5460

tgcttcctgt acagcctgca gaaccatcag ccaattaaac ctctttttctg tataaattac 5520

cagtcttgag tatctctttа cagcagtgtg agaacggact aatacaaggg tctccaaaat 5580

tccaagttta tgtattcttt cttgccaaat agcaggtatt taccataaat cctgtccttа 5640

ggtcaaacaa ccttgatggc atcgtacttc aattgtctta cacattcctt ctgaatgact 5700

cctcccctat ggcatataag ccctgggtct tgggggataa tggcagaggg gtccaccatc 5760

ttgtctggct gccacctgag acacggacat ggcttctgtt ggtaagtctc tattaaatgt 5820

ttctttctaa gaaactggat ttgtcagctt gtttctttgg cctctcagct tcctcagact 5880

ttggggtagg ttgcacaacc ctgcccacca cgaaacaaac gtttaatatg ataaatatgg 5940

atagatataa tccacataaa taaaagctct tggagggccc tcaataattg ttaagagtgt 6000

aaatgtgtcc aaagatggaa aatgtttgag aactactgtc ccagagattt tcctgagttc 6060

tagagtgtgg gaatatagaa cctggagctt ggcttcttcc gcctagaatc aggagtatgg 6120

ggctgaagtc tgaagcttgg cttcagcagt ttggggttgg cttccggagc acatatttga 6180

catgttgcga ctgtgatttg gggtttggta tttgctctga atcctaatgt ctgtccttga 6240

ggcatctaga atctgaaatc tgtggtcaga attctattat cttgagtagg acatctccag 6300

tcctggttct gccttctagg gctggagtct gtagtcagtg acccggtctg gcatttcaac 6360

ttcatataca gtgggctatc ttttggtcca tgtttcaacc aaacaaccga ataaaccatt 6420

agaacctttc cccacttccc tagctgcaat gttaaacctа ggatttctgt ttaataggtt 6480

catatgaata atttcagcct gatccaactt tacattcctt ctaccgttat tctacacccа 6540

ccttaaaaat gcattcccaa tatatttccct ggatttctacc tatatatggt aatcctggct 6600

ttgccagttt ctagtgcatt aacatacctg atttacattc ttttacttta aagtggaaat 6660

aagagtccct ctgcagagtt caggagttct caagatggcc cttacttctg acatcaattg 6720

agatttcaag ggagtcgcca agatcatcct caggttcagt gattgctggt agccctcata 6780

【図６Ｅ】

FIGURE 6E

taactcaatg aaagctgtta tgctcatggc tatggtttat tacagcaaaa gaatagagat 6840
gaaaatctag cagcggaaga gttgcatggg gcaaagacaa ggagagctcc aagtgcagag 6900
attcctgttg ttttctcccc gtggtgtcat ggaaagcagt atcttctcca tacaatgatg 6960
tgtgataata ttcagtgtat tgccaatcag ggaactcaac tgagccttga ttatattgga 7020
gcttggttgc acagacatgt cgaccacctt catggctgaa ctttagtact tagcccctcc 7080
agacgtctac agctgatagg ctgtaaccca acattgtcac cataaatcac attgttagac 7140
tatccagtgt ggccccaagct ccccgtgtaaa cacaggcact ctaaacaggc aggatatttc 7200
aaaagcttag agatgacctc ccaggagctg aatgcaaaga cctggcctct ttgggcaagg 7260
agaatccttt accgcacact ctccttcaca gggttattgt gaggatcaaa tgtggtcatg 7320
tgtgtgagac acccagcacat gtctggctgt ggagagtgac ttctatgtgt gctaacattg 7380
ctgagtgcta agaaaagtatt aggcatggct ttcagcactc acagatgctc atctaatcct 7440
cacaacatgg ctacagggtg ggcactacta gcctcatttg acagaggaaa ggactgtgga 7500
taagaagggg gtgaccaata ggtcagagtc attctggatg caaggggctc cagaggacca 7560
tgattagaca ttgtctgcag agaaattatg gctggatgtc tctgccccgg aaaggggcat 7620
gcactttcct tgacccccta tatcagatct tgactttgag gttatctcag acttcctcta 7680
tgataccagg agcccatcat aatctctctg tgtcctatcc cattcctcag tcttactgcc 7740
cactcttccc agctccatct ccagctggcc aggtgtagcc acagtaccta actctttgca 7800
gagaactata aatgtgtatc ctacagggga gaaaaaaaaa aagaactctg aaagagctga 7860
cattttaccg acttgcaaac acataagcta acctgccagt tttgtgctgg tagaactcat 7920
gagactcctg ggtcagaggc aaaagatttt attacccaca gctaaggagg cagcatgaac 7980
tttgtgttca catttgttca ctttgccccc caattcatat gggatgatca gagcagttca 8040
ggtggatgga cacaggggtt tgtggcaaag gtgagcaacc taggcttaga aatcctcaat 8100
cttctaagaa ggtactagca aacttgtcca gtctttgtat ctgacggaga tattatcttt 8160
ataattgggt tgaaagcaga cctactctgg aggaacatat tgtatttatt gtcctgaaca 8220
gtaaacaaat ctgctgtaaa atagacgtta actttattat ctaaggcagt aagcaaacct 8280
agatctgaag gcgataccat cttgcaaggc tatctgctgt acaaatatgc ttgaaaagat 8340
ggtccagaaa agaaaacggt attattgcct ttgctcagaa gacacacaga aacataagag 8400
aaccatggaa aattgtctcc caacactgtt cacccagagc cttccactct tgtctgcagg 8460
acagtcttaa catcccatca ttagtgtgtc taccacatct ggcttcaccg tgcctaacca 8520

【図６Ｆ】

FIGURE 6F

agatttctag gtccagttcc ccaccatgtt tggcagtgcc ccactgccaa ccccagaata 8580
agggagtgct cagaattccg aggggacatg ggtggggatc agaacttctg ggcttgagtg 8640
cagaggggcc ccatactcct tggttccgaa ggaggaagag gctggaggtg aatgtccttg 8700
gaggggagga atgtgggttc tgaactctta aatccccaag ggaggagact ggtaaggtcc 8760
cagcttccga ggtactgacg tgggaatggc ctgagaggtc taagaatccc gtatcctcgg 8820
gaaggagggg ctgaaattgt gaggggttga gttgcagggg tttgttagct tgagactcct 8880
tggtgggccc ctgggaagca aggactggaa ccattggctc cagggtttgg tgtgaaggta 8940
atgggatctc ctgattctca aagggtcaga ggactgagag ttgcccatgc tttgatcttt 9000
ccatctactc cttactccac ttgagggtaa tcacctactc ttctagttcc acaagagtgc 9060
gcctgcgcga gtataatctg cacatgtgcc atgtcccgag gcctggggca tcatccactc 9120
atcattcagc atctgcgcta tgcgggcgag gccggcgcca tgacgtcatg tagctgcgac 9180
tatccctgca gcgcgcctct cccgtcacgt cccaaccatg gagctgtgga cgtgcgtccc 9240
ctggtggatg tggcctgcgt ggtgccaggc cggggcctgg tgtccgataa agatcctaga 9300
accacaggaa accaggactg aaaggtgcta gagaatggcc atatgtcgct gtccatgaaa 9360
tctcaaggac ttctgggtgg agggcacagg agcctgaact tacgggtttg ccccagtcca 9420
ctgtcctccc aagtgagtct cccagatacg aggcactgtg ccagcatcag cttcatctgt 9480
accacatctt gtaacagggа ctacccagga ccctgatgaa caccatggtg tgtgcaggaa 9540
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ggaggaggct gtggactggc tcgagaagtg ggatgtggtt gtgtttgatt tcctttggcc 9660
agataaagtg ctggatatag cattgaaaac ggagtatgaa gaccagttag aatggagggt 9720
caggttggag ttgagttaca gatggggtaa aattctgctt cggatgagtt tggggattgg 9780
caatctaaag gtggtttggg atggcatggc tttgggatgg aaataggttt gttttatgt 9840
tggctgggaa gggtgtgggg attgaattgg ggatgaagta ggtttagttt tggagataga 9900
atacatggag ctggctattg catgcgagga tgtgcattag tttgtttga tctttaaata 9960
aaggaggcta ttagggttgt cttgaattag attaagttgt gttgggttga tgggttgggc 10020
ttgtgggtga tgtggttgga ttgggctgtg ttaaattggt ttgggtcagg ttttggttga 10080
ggttatcatg gggatgagga tatgcttggg acatggattc aggtggttct cattcaagct 10140
gaggcaaatt tcctttcaga cggtcattcc agggaacgag cggttgtgtg ggggaaatca 10200

【図６Ｇ】

FIGURE 6G

ggccactggc tgtgaatatc cctctatcct ggtcttgaat tgtgattatc tatgtccatt 10260
ctgtctcctt cactgtactt ggaattgatc tggtcattca gctggaaatg ggggaagatt 10320
ttgtcaaatt cttgagacac agctgggtct ggatcagcgt aagccttcct tctggtttta 10380
ttgaacagat gaaatcacat ttttttttte aaaatcaceg aaatcttata gagttaacag 10440
tggactctta taataagagt taacaccagg actcttattc ttgattcttt tctgagacac 10500
caaaatgaga tttctcaatg ccaccctaat tcttttttct ttttttttct tttttgagac 10560
acagtctggg tcttttgctc tgtcactcag gctggagcgc agtggtgtga tcatagctca 10620
ctgaacctt gacctcctgg acttaaggga tcctcctgct tcagcctcct gagtagatgg 10680
ggctacaggt gcttgccacc acacctggct aattaaattt ttttttttt tttgtagaga 10740
aagggtctca ctttgttgcc ctggctgatc ttgaacttct gacttcaagt gattcttcag 10800
ccttggactc ccaaagcact gggattgctg gcatgagcca ctcaccgtgc ctggcttgca 10860
gcttaatctt ggagtgtcta aacctggctc ctgatagcta gacatttcag tgagaaggag 10920
gcattggatt ttgcatgagg acaattctga cctaggaggg caggtcaaca ggaatccccg 10980
ctgtacctgt acgttgtaca ggcatggaga atgaggagtg aggaggccgt accggaaccc 11040
catattgttt agtggacatt ggattttgaa ataataggga acttggtctg ggagagtcat 11100
atttctggat tggacaatat gtggtatcac aaggttttat gatgagggag aaatgtatgt 11160
ggggaaccat tttctgagtg tggaagtgca agaatcagag agtagctgaa tgccaacgct 11220
tctatttcag gaacatggta agttggaggt ccagctctcg ggctcagacg ggtataggga 11280
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aaggaagtac ttgggactta ggcacatgag actttgtatt gaaaatcaat gattgggggt 11400
ggccgtggtg ctcacgcctg taatctcatc actttgggag accgaagtgg gaggatggct 11460
tgatctcaag agttggacac cagcctaggc aacatggcca gaccctctct ctacaaaaaa 11520
attaaaaatt agctggatgt ggtggtgcat gcttgtggtc tcagctatcc tggaggctga 11580
gacaggagaa tcggttgagt ctgggagttc aaggctacag ggagctgcga tcacgccgct 11640
gcactccagc ctgggaaaca gagtgagact gtctcagaat ttttttaaaa aagaatcagt 11700
gatcatccca accectgttg ctgttcatcc tgagcctgcc ttctctggct ttgttcccta 11760
gatcacatct ccatgatcca taggccctgc ccaatctgac ctcaacccgt gggaatgcct 11820
ccagactgat ctagtatgtg tggaacagca agtgctggct ctcccttccc ttccacaget 11880
ctgggtgtgg gagggggttg tccagcctcc agcagcatgg ggagggcctt ggtcagcatc 11940

【図６Ｈ】

FIGURE 6H

taggtgccaa cagggcaagg gcggggtcct ggagaatgaa ggctttatag ggctcctcag 12000
ggaggccccc cagccccaaa ctgcaccacc tggccgtgga caccggt 12047

【図7】

FIGURE 7

cgagcggccc ctcagcttcg gcgcccagcc ccgcaaggct ccoggtgacc actagagggc 60
gggaggagct cctggccagt ggtggagagt ggcaaggaag gaccctaggg ttcatcggag 120
cccaggttta ctcccttaag tggaaatttc ttccccccact cctccttggc tttctccaag 180
gagggaaccc aggctgctgg aeagtccggc tggggcgggg actgtgggtt cagggggaga 240
cggggtgtgg aacgggacag ggagcggtta gaagggtggg gctattccgg gaagtggtgg 300
ggggagggag cccaaaacta gcacctagtc cactcattat ccagccctct tatttctggg 360
ccgctctgct tcagtggacc cgggyagggc ggggaagtgg agtgggagac ctagggggtgg 420
gcttcccgac cttgctgtac aggacctcga cctagctggc tttgttcccc atccccacgt 480
tagttgttgc cctgaggcta aaactagagc ccagggycce caagttccag actgcccctc 540
ccccctcccc cggagcccgg gagtggttgg tgaaagggge aggccagctg gagaacaaac 600
gggtagtcag ggggttgagc gattagagcc cttgtaccct acccaggaat ggttggggag 660
gaggaggaag aggtaggagg taggggaggg ggcgggggttt tgtcacctgt cacctgctcg 720
ctgtgcctag ggcgggcggg cggggagtgg ggggaccggt ataaagcggt aggcgcctgt 780
gcccgctcca cctctcaagc agccagcgcc tgcctgaatc tgttctgccc cctccccacc 840
catttcacca ccaccatg 858

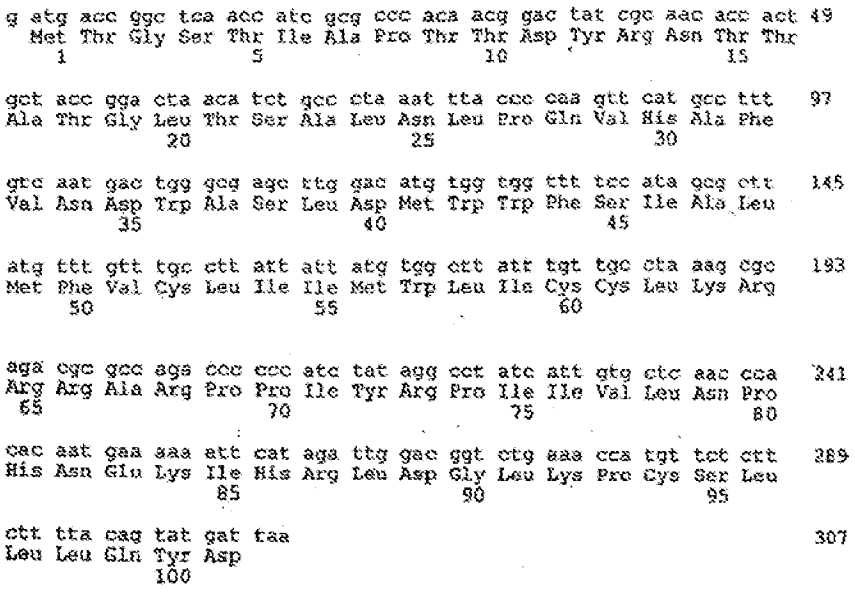
【図8】

FIGURE 8

aagcttccac aagtgcattt agcctctcca gtattgctga tgaatccaca gttcaggttc 60
aatggcgttc aaaacttgat caaaaatgac cagactttat attcttacac caacatctat 120
ctgattggag gaatggataa tagtcatcat gtttaaacat ctaccattcc agttaagaaa 180
atatgatagc atcttgttct tagtcttttt cttaataggg acataaagcc cacaaataaa 240
aatatgcctg aagaatggga caggcattgg gcattgtcca tgcctagtaa agtactccaa 300
gaacctattt gtatactaga tgacacaatg tcaatgtctg tgtacaactg ccaactggga 360
tgcaagacac tgcccatgcc aatcatcctg aaaagcagct ataaaaagca ggaagctact 420
ctgcaccttg tcagtgaggt ccagatacct acag 454

【図９】

FIGURE 9


g atg acc ggc tca acc atc gcg ccc aca acg gac tat cgc aac acc act 49
Met Thr Gly Ser Thr Ile Ala Pro Thr Thr Asp Tyr Arg Asn Thr Thr
1 5 10 15
gct acc gga cta aca tct gcc cta aat tta ccc caa gtt cat gcc ttt 97
Ala Thr Gly Leu Thr Ser Ala Leu Asn Leu Pro Gln Val His Ala Phe
20 25 30
gtc aat gac tgg gcg agc ttg gac atg tgg tgg ttt tcc ata gcg ctt 145
Val Asn Asp Trp Ala Ser Leu Asp Met Trp Trp Phe Ser Ile Ala Leu
35 40 45
atg ttt gtt tgc ctt att att atg tgg ctt att tgt tgc cta aag cgc 193
Met Phe Val Cys Leu Ile Ile Met Trp Leu Ile Cys Cys Leu Lys Arg
50 55 60
aga cgc gcc agg ccc ccc atc tat agg cct atc att gtg ctc aac cca 241
Arg Arg Ala Arg Pro Pro Ile Tyr Arg Pro Ile Ile Val Leu Asn Pro
65 70 75 80
cac aat gaa aaa att cat aga ttg gac ggt ctg aaa cca tgt tct ctt 289
His Asn Glu Lys Ile His Arg Leu Asp Gly Leu Lys Pro Cys Ser Leu
85 90 95
ctt tta cag tat gat taa 307
Leu Leu Gln Tyr Asp
100

【国際調査報告】

INTERNATIONAL SEARCH REPORT

Intl. Internat Application No
PCT/US 99/20718

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/86 C12N5/10 A61K48/00

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 7 C12N A61K

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.
Y	US 5 698 443 A (HENDERSON DANIEL ROBERT ET AL) 16 December 1997 (1997-12-16) cited in the application the whole document	1-17, 23-28
Y	SEMENZA G. L. ET AL.: "Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 51, 1996, pages 32529-32537, XP002129236 the whole document, especially Fig. 10	1-10, 14-17, 23-28

-/--

[X] Further documents are listed in the continuation of box C. [X] 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

Date of the actual completion of the international search	Date of mailing of the international search report
31 January 2000	11/02/2000

Name and mailing address of the ISA	Authorized officer
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	Mandl, B

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 99/20718

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 13508 A (DANA FARBER CANCER INST INC ;KAELIN WILLIAM JR (US); FINE HOWARD A) 2 April 1998 (1998-04-02) page 3, last paragraph - paragraph 2 page 9, line 1 - line 11	1-8,11, 12, 14-17, 23-28
Y	WO 98 06864 A (US HEALTH ;MOONEN CHRIT (US)) 19 February 1998 (1998-02-19) page 10, line 36 -page 12, line 8	1-8, 13-17, 23-28
A	EP 0 845 537 A (CHIRON CORP) 3 June 1998 (1998-06-03) page 22, line 31 - line 34	18-22
A	WO 96 17053 A (GENETIC THERAPY INC ;HALLENBECK PAUL L (US); CHANG YUNG NIEN (US);) 6 June 1996 (1996-06-06) cited in the application the whole document	1-28
A	RINSCH C. ET AL.: "A GENE THERAPY APPROACH TO REGULATED DELIVERY OF ERYTHROPOIETIN AS A FUNCTION OF OXYGEN TENSION." HUMAN GENE THERAPY, vol. 8, no. 16, November 1997 (1997-11), pages 1881-1889, XP000867701 the whole document	9,10
A	ZWICKER J. ET AL.: "CELL CYCLE REGULATION OF THE CYCLIN A, CDC25C AND CDC2 GENES IS BASED ON A COMMON MECHANISM OF TRANSCRIPTIONAL REPRESSION" EMBO JOURNAL, vol. 14, no. 18, 1 January 1995 (1995-01-01), pages 4514-4522, XP002038970 ISSN: 0261-4189 the whole document	11,12

1

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/20718

Box I Observations where certain claims were found unsearchable (Continuation of item 1 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. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 27 and 28, as far as in vivo application is concerned, are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
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:

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 II Observations where unity of invention is lacking (Continuation of item 2 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.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte ernal Application No
PCT/US 99/28718

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(81)指定国 EP(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OA(BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG), AP(GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), EA(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, 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, 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, ZA, ZW

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4J002 AE051 CH052 GB04
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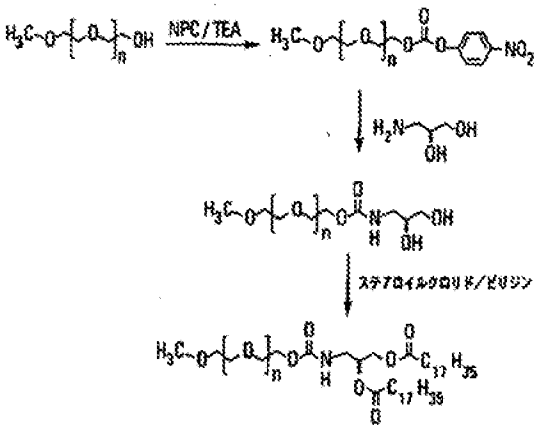
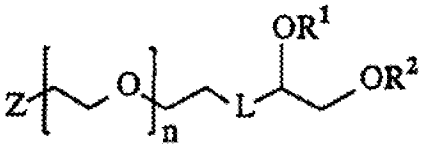
(54)【発明の名称】　中性リポポリマーとそれを含有するリポソーム組成物

(57)【要約】
ＰＥＧ置換中性リポポリマーを含有するリポソームは、慣用的な負荷電ＰＥＧ置換リン脂質を組み込むリポソームと同様な循環時間を生じる。非荷電リポポリマーの使用はさらに、細胞表面との相互作用と、リポソームからの荷電物質、特にカチオン性薬物の漏出減少とに関する利益を提示することができる。これらのリポポリマーは式（Ｉ）によって示される。この式中、R^1とR^2の各々は約８～約２４個の炭素原子を有するアルキル又はアルケニル鎖であり；ｎは約１０～約３００であり；Ｚはヒドロキシ，アルコキシ，ベンジルオキシ，カルボン酸エステル，スルホン酸エステル，アルキル又はアリールカーボネート，アミノ及びアルキルアミノから成る群から選択され；結合Ｌは（ｉ）－Ｘ－（Ｃ＝Ｏ）－Ｙ－CH_2－，（ｉｉ）－Ｘ－（Ｃ＝Ｏ）－及び（ｉｉｉ）－Ｘ－CH_2－から成る群から選択され，この場合，ＸとＹは酸素，ＮＨ及び直接結合から独立的に選択される。



【特許請求の範囲】

【請求項１】 式：



〔式中、R^1とR^2の各々は約８～約２４個の炭素原子を有するアルキル又はアルケニル鎖であり；ｎ＝約１０～約３００；Ｚはヒドロキシ、アルコキシ、ベンジルオキシ、カルボン酸エステル、スルホン酸エステル、アルキル又はアリールカーボネート、アミノ及びアルキルアミノから成る群から選択され；Ｌは（ｉ）－Ｘ－（Ｃ＝Ｏ）－Ｙ－CH_2－、（ｉｉ）－Ｘ－（Ｃ＝Ｏ）－及び（ｉｉｉ）－Ｘ－CH_2－から成る群から選択され、この場合、ＸとＹは酸素、ＮＨ及び直接結合から独立的に選択される〕で示される中性リポポリマー約１モル％～約１０モル％と、残部のベシクル形成脂質とを含むリポソーム組成物。

【請求項２】 Ｘが酸素であり、Ｙが窒素である、請求項１記載の組成物。

【請求項３】 Ｌがカルバメート結合、エステル結合又はカーボネート結合である、請求項１記載の組成物。

【請求項４】 Ｌが－Ｏ－（Ｃ＝Ｏ）－Ｎ－CH_2－（カルバメート結合）である、請求項３記載の組成物。

【請求項５】 Ｚがヒドロキシ又はメトキシである、請求項１記載の組成物。

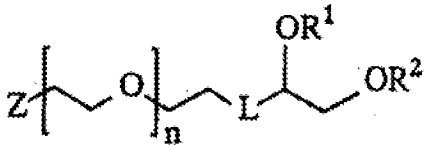
【請求項６】 約３モル％～約６モル％の中性リポポリマーをさらに含む、請求項１記載の組成物。

【請求項７】 R^1とR^2の各々が約８～約２４個の炭素原子を有する非分枝アルキル又はアルケニル鎖である、請求項１記載の組成物。

【請求項８】 R^1とR^2の各々が$C_{17}H_{35}$である、請求項６記載の組成物。

【請求項９】 ｎが約２０～約１１５である、請求項１記載の組成物。

【請求項１０】 ベシクル形成脂質を含むリポソームの循環時間を延長する方法であって、リポソーム中にベシクル形成脂質と共に、式：



［式中、R^1とR^2の各々は約８～約２４個の炭素原子を有するアルキル又はアルケニル鎖であり；ｎ＝約１０～約３００；Ｚはヒドロキシ、アルコキシ、ベンジルオキシ、カルボン酸エステル、スルホン酸エステル、アルキル又はアリールカーボネート、アミノ及びアルキルアミノから成る群から選択され；Ｌは（ｉ）－Ｘ－（Ｃ＝Ｏ）－Ｙ－CH_2－、（ｉｉ）－Ｘ－（Ｃ＝Ｏ）－及び（ｉｉｉ）－Ｘ－CH_2－から成る群から選択され、この場合、ＸとＹは酸素、ＮＨ及び直接結合から独立的に選択される］で示される中性リポポリマー約１モル％～約１０モル％を組み込むことを含む前記方法。

【請求項１１】　Ｘが酸素であり、Ｙが窒素である、請求項１０記載の方法。

【請求項１２】　Ｌがカルバメート結合、エステル結合又はカーボネート結合である、請求項１０記載の方法。

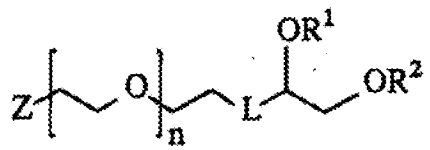
【請求項１３】　Ｌが－Ｏ－（Ｃ＝Ｏ）－Ｎ－CH_2－（カルバメート結合）である、請求項１２記載の方法。

【請求項１４】　Ｚがヒドロキシ又はメトキシである、請求項１０記載の方法。

【請求項１５】　約３モル％～約６モル％の中性リポポリマーを組み込む、請求項１０記載の方法。

【請求項１６】　ｎが約２０～約１１５である、請求項１０記載の方法。

【請求項１７】　式：



［式中、R^1とR^2の各々は約８～約２４個の炭素原子を有するアルキル又はアル

ケニル鎖であり；ｎ＝約１０～約３００；Ｚはヒドロキシ、アルコキシ、ベンジルオキシ、カルボン酸エステル、スルホン酸エステル、アルキル又はアリールカーボネート、アミノ及びアルキルアミノから成る群から選択され；Ｌは（ｉ）－Ｘ－（Ｃ＝Ｏ）－Ｙ－CH_2－、（ｉｉ）－Ｘ－（Ｃ＝Ｏ）－及び（ｉｉｉ）－Ｘ－CH_2－から成る群から選択され、この場合、ＸとＹは酸素、ＮＨ及び直接結合から独立的に選択される］で示される中性リポポリマー。

【請求項１８】　Ｘが酸素であり、Ｙが窒素である、請求項１７記載のリポポリマー。

【請求項１９】　Ｌがカルバメート結合、エステル結合又はカーボネート結合である、請求項１７記載のリポポリマー。

【請求項２０】　Ｌが－Ｏ－（Ｃ＝Ｏ）－Ｎ－CH_2－（カルバメート結合）である、請求項１９記載のリポポリマー。

【請求項２１】　R^1とR^2の各々が約８～約２４個の炭素原子を有する非分枝アルキル又はアルケニル鎖である、請求項１７記載のリポポリマー。

【請求項２２】　R^1とR^2の各々が$C_{17}H_{35}$である、請求項２１記載のリポポリマー。

【請求項２３】　Ｚがヒドロキシ又はメトキシである、請求項１７記載のリポポリマー。

【請求項２４】　ｎが約２０～約１１５である、請求項１７記載のリポポリマー。

【発明の詳細な説明】

【０００１】

（発明の分野）

本発明は、ＰＥＧ置換中性リポポリマーと、循環時間の長いリポソームへのそれらの使用とに関する。これらのリポポリマーを含有するリポソームは、慣用的な負荷電ＰＥＧ置換リン脂質を組み込むリポソームと比較した場合に同様な血液循環時間を生じる。

【０００２】

（関連出願へのクロスーリファレンス）

１９９９年７月１４日に米国特許庁に出願された、“中性リポポリマーとそれを含有するリポソーム組成物”なる名称の米国暫定特許出願第６０／１４３，８１０号に記載された完全な開示は、本明細書に援用される。これらの出願は共通に所有される。

【０００３】

（発明の背景）

リポソームは多様な治療目的のために、特に、治療剤のリポソーム製剤を全身投与することによって、これらの治療剤を標的細胞に運搬するために用いられる。有利なことには、リポソーム－薬物製剤は例えば、制御された薬物放出を包含する、改良された薬物－デリバリー特性(drug-delivery properties)の可能性を提供する。リポソームが標的の領域、細胞又は部位に達するためには、長い循環時間が必要である。特に、標的の領域、細胞又は部位が注射部位の近くに存在しない場合に、このことが必要である。例えば、リポソームを全身投与する場合に、リポソームを親水性作用剤によって、例えば、ポリエチレングリコール（ＰＥＧ）のような親水性ポリマー鎖のコーティングによって被覆して、リポソームの血液循環寿命を延長することが望ましい。このような表面修飾リポソームは一般的に“長時間循環する”又は“立体的に安定化した”リポソームと呼ばれる。

【０００４】

リポソームの最も一般的な表面修飾は、典型的に約１０００ダルトン（Ｄａ）から約５０００Ｄａまでの分子量を有するＰＥＧ鎖をリポソームを構成する脂質

の約５モル％に取り付けることである（例えば、Ｓｔｅａｌｔｈ Ｌｉｐｏｓｏｍｅｓ，ＣＲＣ Ｐｒｅｓｓ，Ｌａｓｉｃ，Ｄ．とＭａｒｔｉｎ，Ｆ．編集，Ｂｏｃａ Ｒａｔｏｎ，ＦＬ，（１９９５）と、これに引用された参考文献参照）。このようなリポソームによって示される薬物動態は、血液から迅速に除去されて、肝臓と脾臓に蓄積される傾向がある非表面修飾リポソームに比べて、単核食細胞系（ＭＰＳ）を介した肝臓と脾臓によるリポソームの取り込みの投与量非依存的減少と、顕著に長い循環時間とを特徴とする。

【０００５】

最も一般的に用いられる、商業的に入手可能なＰＥＧ置換リン脂質は、極性ヘッド基において負に荷電している、ホスファチジルエタノールアミン（ＰＥ）、通常はＤＳＰＥ（ジステアロイルホスファチジルエタノールアミン）に基づくものである。リポソームにおける負の表面電荷は、幾つかの見地において、例えば、細胞との相互作用において（例えば、Ｍｉｌｌｅｒ等，Ｂｉｏｃｈｅｍｉｓｔｒｙ，３７：１２８７５－１２８８３（１９９８）参照）及び薬物の漏出が起こりうるカチオン性薬物のデリバリーにおいて（例えば、Ｗｅｂｂ等，Ｂｉｏｃｈｅｍ．Ｂｉｏｐｈｙｓ．Ａｃｔａ，１３７２：２７２－２８２（１９９８）参照）不利である可能性がある。

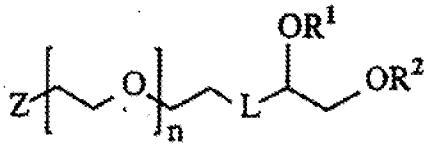
【０００６】

したがって、効果的に脂質二重層中に組み込まれて、リポソームに長い血液循環時間を与える非荷電ＰＥＧ誘導体化脂質を提供することは有益であると考えられる。このような脂質が低毒性であり、容易に製造されることが理想的である。

【０００７】

（発明の概要）

１態様において、本発明は、約１モル％～約１０モル％の中性リポポリマーを有するリポソーム組成物であって、該中性リポポリマーが式：



〔式中、R^1とR^2の各々は約８～約２４個の炭素原子を有するアルキル又はアルケニル鎖であり；ｎ＝約１０～約３００；Ｚはヒドロキシ、アルコキシ、ベンジルオキシ、カルボン酸エステル、スルホン酸エステル、アルキル又はアリールカーボネート、アミノ及びアルキルアミノから成る群から選択され；Ｌは（ｉ）－Ｘ－（Ｃ＝Ｏ）－Ｙ－CH_2－、（ｉｉ）－Ｘ－（Ｃ＝Ｏ）－及び（ｉｉｉ）－Ｘ－CH_2－から成る群から選択され、この場合、ＸとＹは酸素、ＮＨ及び直接結合から独立的に選択される〕によって示される前記リポソーム組成物を包含する。好ましくは、該組成物は約３モル％～約６モル％の中性リポポリマーを包含する。

【０００８】

他の態様では、Ｌは例えばカルバメート結合、エステル結合又はカーボネート結合のような加水分解可能な結合である。さらに他の態様では、Ｚはヒドロキシ又はメトキシである。好ましくは、R^1とR^2は非分枝状である。１態様では、R^1とR^2は両方ともステアリル基（$C_{18}H_{36}$）である。他の態様では、ＰＥＧ基の分子量が約１０００Ｄａ～約５０００Ｄａになるように、ｎの値は好ましくは約２０～約１１５である。

【０００９】

本発明はまた、上記に示したような式を有する中性リポポリマー約１モル％～約１０モル％をリポソーム中に組み入れることによって、ベシクル形成脂質を含有するリポソームの血液循環時間を延長する方法をも提供する。本発明はさらに、この式によって表されるリポポリマーを提供する。

【００１０】

（発明の詳細な説明）

１．定義

本明細書で用いる限り、“中性”リポポリマーなる用語は、非荷電である、即ち、イオン性を有さないリポポリマーを意味する。

【００１１】

“ベシクル形成脂質”は、疎水性で極性のヘッド基部分を有する両親媒性脂質を意味する。このようなベシクル形成脂質は、リン脂質によって例証されるよう

に、自発的に二重層に形成されることができる、又は安定に脂質二重層中に組み込まれることができ、この場合に疎水性部分が二層膜の内側領域、即ち、単一疎水性領域に接触し、極性ヘッド基部分が膜の外側表面、即ち、極性表面方向に配向される。このタイプのベシクル形成脂質のクラスは典型的に１つ又は２つの疎水性アシル炭化水素鎖又はステロイド基を包含し、極性ヘッド基において化学的反応基（例えば、アミン、酸、エステル、アルデヒド又はアルコール）を含有することができる。このクラスには、例えばホスファチジルコリン（ＰＣ）、ホスファチジルエタノールアミン（ＰＥ）、ホスファチジン酸（ＰＡ）、ホスファチジルイノシトール（ＰＩ）及びスフィンゴミエリン（ＳＭ）のようなリン脂質が包含され、これらにおいて、２本の炭化水素鎖は典型的に約１４～約２２炭素原子の長さであり、変化する不飽和度を有する。他のベシクル形成脂質は例えばセレブロシド及びガングリオシドのような糖脂質と、例えばコレステロールのようなステロールとを包含する。本明細書に述べる組成物のために、例えばＰＣ及びＰＥのようなリン脂質、コレステロール、及び本明細書に述べる中性リン脂質が好ましい化合物である。

【００１２】

“アルキル”は、炭素と水素とを含有し、分枝鎖又は直鎖であることができる完全飽和一価ラジカルを意味する。アルキル基の例はメチル、エチル、ｎ－ブチル、ｔ－ブチル、ｎ－ヘプチル及びイソプロピルである。“低級アルキル”は、メチル、エチル、ｎ－ブチル、ｉ－ブチル、ｔ－ブチル、イソアミル、ｎ－ペンチル及びイソペンチルによって例証されるような、約１～約６炭素原子のアルキルラジカルを意味する。

【００１３】

“アルケニル”は、炭素と水素とを含有し、分枝鎖又は直鎖であることができ、少なくとも１つの二重結合を含有する一価ラジカルを意味する。

【００１４】

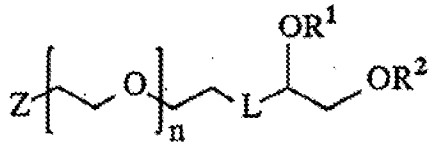
略号：ＰＥＧ：ポリエチレングリコール；ｍＰＥＧ：メトキシ末端ポリエチレングリコール；Ｃｈｏｌ：コレステロール；ＰＣ：ホスファチジルコリン；ＰＨＰＣ：部分的水素化ホスファチジルコリン；ＰＨＥＰＣ：部分的水素化卵ホスフ

ァチジルコリン；ＨＳＰＣ：水素化ダイズホスファチジルコリン；ＤＳＰＥ：ジステアロイルホスファチジルエタノールアミン；ＤＳＰ又はＰＥＧ－ｃ－ＤＳ：ジステアロイル（カルバメート連結）ＰＥＧ；ＡＰＤ：１－アミノ－２，３－プロパンジオール；ＤＩＴＡ：ジエチレンテトラミン五酢酸；Ｂｎ：ベンジル。

【００１５】

ＩＩ．中性リポポリマー

本発明のＰＥＧ置換中性リポポリマーは以下に示す構造を有する：



式中、R^1とR^2の各々は約８～約２４個の炭素原子を有するアルキル又はアルケニル鎖であり；ｎは約１０～約３００であり；Ｚはヒドロキシ、アルコキシ、ベンジルオキシ、カルボン酸エステル、スルホン酸エステル、アルキル又はアリールカーボネート、アミノ及びアルキルアミノから成る群から選択され；Ｌは（ｉ）－Ｘ－（Ｃ＝Ｏ）－Ｙ－CH_2－、（ｉｉ）－Ｘ－（Ｃ＝Ｏ）－及び（ｉｉｉ）－Ｘ－CH_2－から成る群から選択され、この場合、ＸとＹは酸素、ＮＨ及び直接結合から独立的に選択される。

【００１６】

リポポリマーは、立体的に安定化したリポソームにしばしば用いられる、例えばＰＥＧ－ＤＳＰＥのような、ＰＥＧ－リン脂質の荷電ホスフェート結合の代わりに中性結合（Ｌ）を包含する。この中性結合は典型的に、カルバメート、エステル、アミド、カーボネート、尿素、アミン及びエーテルから選択される。ｉｎｖｉｖｏで一定の循環時間後にＰＥＧ鎖を除去することが望ましい用途では、例えばカルバメート、カーボネート及びエステルのような加水分解可能な又は他のやり方で切断可能な結合が好ましい。この特徴は、リポソームがその標的に達した後に、薬物を放出する又は細胞への取り込みを促進することに有用でありうる（Ｍａｒｔｉｎ等，米国特許第５，８９１，４６８号；Ｚａｌｉｐｓｋｙ等，ＰＣＴ公開第ＷＯ９８／１８８１３号（１９９８））。

【００１７】

連結基(linking group)に取り付いたＰＥＧ基は好ましくは、約１０００Ｄａ～約１５０００Ｄａの分子量を有する；即ち、この場合には、ｎは約２０～約３４０である。さらに好ましくは、該分子量は約１０００Ｄａ～約１２０００Ｄａ（ｎ＝約２０～約２７５）であり、最も好ましくは、約１０００Ｄａ～約５０００Ｄａ（ｎ＝約２０～約１１５）である。R^1とR^2は典型的に約８～約２４炭素原子長さであり、好ましくは約１６～約２０炭素原子長さである。最も好ましくは、ＣＯＯＲがステアリル基であるように、$R^1=R^2=C_{17}H_{35}$である。

【００１８】

上述したように、非荷電脂質のリポソーム中への取り込みは例えば封入カチオン性薬物の漏出の減少のような有意な利益を提示しうる。さらに、他の利益は、リポソーム表面と標的細胞及びＲＥＳとの相互作用のモジュレーティングにおけるより大きなフレキシビリティである（Ｍｉｌｌｅｒ等，Ｂｉｏｃｈｅｍｉｓｔｒｙ，３７：１２８７５－１２８８３（１９９８））。ＰＥＧ置換合成セラミドは、立体的に安定化したリポソームの非荷電成分として用いられている（Ｗｅｂｂ等，Ｂｉｏｃｈｅｍ．Ｂｉｏｐｈｙｓ．Ａｃｔａ，１３７２：２７２－２８２（１９９８））；しかし、これらの分子は複雑であり、製造するのに費用がかかり、これらの分子は一般的にリン脂質二重層並びにジアシルグリセロリン脂質中にパックされない。

【００１９】

リポポリマーは標準合成方法を用いて調製することができる。例えば、カルバメート連結化合物（Ｌ＝－Ｏ－（Ｃ＝Ｏ）－ＮＨ－CH_2－）は、図１に示すように、ｍＰＥＧ（メトキシ－ＰＥＧ）の末端ヒドロキシルをｐ－ニトロフェニルクロロホルメートと反応させて、ｐ－ニトロフェニルカーボネートを得ることによって調製される。この生成物を次に１－アミノ－２，３－プロパンジオールと反応させて、中間体のカルバメートを得る。該ジオールのヒドロキシル基をアシル化して、最終生成物を得る。１－アミノ－２，３－プロパンジオールの代わりにグリセロールを用いる同様な合成を用いて、カーボネート連結生成物（Ｌ＝－Ｏ－（Ｃ＝Ｏ）－Ｏ－CH_2－）を製造することができる。カルバメート連結ジ

ステアロイル及びジエコサノイルリポポリマーの調製も実施例１において述べる。

【００２０】

図２Ａに示すように、エーテル連結リポポリマー（Ｌ＝－Ｏ－CH_2－）は、ｍＰＥＧ－ＯＨの末端ヒドロキシルをグリシジルクロリドと反応させ、得られたエポキシドを加水分解し、得られたジオールをアシル化することによって調製される。エステル連結リポポリマー（Ｌ＝－Ｏ－（Ｃ＝Ｏ）－又は－Ｏ－（Ｃ＝Ｏ）－CH_2－）は例えば、図２Ｂに示すように、ｍＰＥＧ－ＯＨをグリセリン酸アセトニド（２，２－ジメチル－１，３－ジオキソラン－４－カルボン酸）又は４炭素同族体、２，２－ジメチル－１，３－ジオキソラン－４－酢酸の活性化誘導体と反応させることによって調製することができる。次に、ジオールを脱保護して、アシル化する。

【００２１】

ｍＰＥＧ－ＯＨの代わりに、Ｚａｌｉｐｓｋｙ等、ＰＣＴ公開第ＷＯ９８／１８８１３号（１９９８）に述べられた方法によって調製された$mPEG-NH_2$を用いる対応反応はを利用して、アミド、尿素又はアミン結合を有するリポポリマー（即ち、これらの場合には、Ｌ＝－ＮＨ－（Ｃ＝Ｏ）－ＮＨ－、－ＮＨ－（Ｃ＝Ｏ）－CH_2－、－ＮＨ－（Ｃ＝Ｏ）－ＮＨ－CH_2－又は－ＮＨ－CH_2－）を調製することができる。

【００２２】

Ｌが－Ｘ－（Ｃ＝Ｏ）－であり、ＸがＯ又はＮＨである化合物は、活性化カルボキシル末端ＰＥＧ（ヒドロキシル末端ＰＥＧの酸化と、例えばニトロフェニルエステルへの転化又はＤＣＣとの反応によるカルボキシル基の活性化とによって調製）と、それぞれ１，２，３－プロパントリオール又は１－アミノ－２，３－プロパンジオールとの反応によって調製することができる。ケト連結化合物（即ち、Ｘが直接結合である場合）は、アルデヒド末端ＰＥＧ（ヒドロキシル末端ＰＥＧの緩和な酸化によって調製）と、例えば１－ブロモ－２，３－プロパンジオールアセトニドのグリニヤール試薬との縮合（図２Ｄ）と、続いての非酸性条件下でのケトンへの酸化と、ジオールへのアセトニドの加水分解とによって調製す

ることができる。各場合に、該ジオールを次に通常のようにアシル化する。

【００２３】

グリセロール部分に結合していないＰＥＧオリゴマーの末端（α末端：上記Ｚ基）は典型的にヒドロキシ又はメトキシであるが、中性リポポリマーへの種々な分子の取り付けを促進するために、及び／又は特定の細胞若しくは組織種類にリポソームを向けること(targeting)に若しくは他のやり方で薬物デリバリーを促進することに用いるために、この末端を当該技術分野において知られた方法に従って官能性化することができる。取り付けられる分子は、例えば、タンパク質、ペプチド、糖類、抗体又はビタミンを包含することができる。実施例２と３は、上記ルートと同様なルートに従ったα－官能性化リポポリマーの調製における工程を述べるが、これらは商業的に入手可能なＰＥＧオリゴマーによって調製され、この場合、α末端は、分子の脂質部分の合成後にヒドロキシルに容易に転化される、例えばｔ－ブチルエーテル又はベンジルエーテルのような基によって置換される。この末端を次に、この場合には、ｐ－ニトロフェニルカーボネートへの転化によって活性化する。

【００２４】

ＩＩＩ．リポソーム薬物動態

ベシクル形成脂質から構成されるリポソーム中に約１～約１０モル％、より好ましくは約３～約６モル％の中性リポポリマーを組み込むことによって、長時間循環リポソームが形成される。例示するために、約３～約５モル％のｍＰＥＧ$_{2000}$－ＤＳＰＥ（ジステアロイルホスファチジルエタノールアミン）又はカルバメート連結リポポリマー、ｍＰＥＧ$_{2000}$－ｃ－ＤＳを実施例４に述べるように調製した。脂質のバランスは１．５：１のモル比率でのＨＳＰＣとコレステロールとから成った。これらのリポソームにマーカー^{125}Ｉ－トリアミニルイヌリンを負荷した。各調製物のサンプルをマウスの尾静脈に注入して、組織分布を種々な時点において、実施例４に述べるように、測定した。血液、肝臓及び脾臓中に存在するレベルを表１～３に提示し、図３Ａ～３Ｃにグラフによって示す。これらのデータはＰＥＧ－ｃ－ＤＳ含有リポソームの薬物動態が、ＰＥＧ－ＤＳＰＥ含有リポソームの薬物動態に非常に類似したことを示す。

【００２５】

表１：血液中のリポソーム分布

時点	注入量の%		
	A	B	C
30分	--	94.8 ± 3.99	89.7 ± 6.94
2時間	85.1 ± 1.99	79.8 ± 3.42	73.0 ± 17.4
6時間	67.1 ± 6.25	54.5 ± 3.05	55.3 ± 2.51
12時間	54.9 ± 6.04	39.7 ± 2.52	44.4 ± 2.52
24時間	14.8 ± 2.81	12.4 ± 2.34	16.6 ± 2.38

【００２６】

表２：肝臓中のリポソーム分布

時点	注入量の%		
	A	B	C
30分	--	2.27 ± 0.13	3.14 ± 0.95
2時間	8.76 ± 2.01	9.42 ± 1.24	11.7 ± 1.74
6時間	21.7 ± 2.55	19.3 ± 1.37	20.8 ± 0.86
12時間	26.6 ± 0.51	26.4 ± 1.99	30.4 ± 1.28
24時間	43.9 ± 2.7	36.6 ± 2.25	42.6 ± 0.48

【００２７】

表３：肝臓中のリポソーム分布

時点	注入量の%		
	A	B	C
30分	--	0.09 ± 0.06	0.23 ± 0.08
2時間	0.96 ± 0.16	0.99 ± 0.09	1.08 ± 0.09
6時間	1.94 ± 0.07	1.96 ± 0.29	2.12 ± 0.13
12時間	3.15 ± 0.31	3.13 ± 0.12	3.35 ± 0.22
24時間	4.69 ± 0.37	3.91 ± 0.31	4.56 ± 0.29

【００２８】

同様な研究によって、両ＰＥＧ脂質の性能をＰＥＧ脂質を含有しない対照製剤と比較した。図４は、ＰＥＧ脂質を含有しない（クロス）、５モル％PEG_{2000}－ＤＳＰＥを含有する(三角）又は５モル％PEG_{2000} －ｃ－ＤＳを含有する(円

)、2：1HSPCリポソームの血液中の保持(retention)を示す。

【0029】

5：55：40のモル比率でmPEG$_{2000}$ -c-DS：PHPC：Cholを含有するリポソームを用いて、さらなる研究を行なった。これらのリポソームをインジウム-DTPA複合体の組み込みによって標識した。24時間目における血液中及び種々な組織中の注入量の%を測定した。結果を表4～6に示す。再び、リポソームは典型的な長時間薬物動態を示し、4時間後に注入量の70%を超える平均保持と、24時間後の30%を超える平均保持を示した。

【0030】

表4：血液中のインジウム注入量の%

動物#	0.0時間	0.5時間	1.0時間	2.0時間	4.0時間	24時間
ラット1	103.7	91.2	82.5	73.8	72.0	33.1
ラット2	97.7	87.7	79.4	78.7	74.4	30.7
ラット3	95.1	83.1	77.8	68.6	64.4	29.8
ラット4	91.9	85.4	78.5	75.6	72.6	33.2
平均値	97.1	86.8	79.6	74.2	70.9	31.7
標準偏差	5.0	3.4	2.1	4.2	4.4	1.7

【0031】

表5：24時間目の組織中の注入量の%

組織	ラット#1	ラット#2	ラット#3	ラット#4	平均値	標準偏差
肝臓	7.5	6.9	6.7	7.2	7.1	0.3
脾臓	4.9	5.4	5.6	4.8	5.2	0.4
心臓	0.4	0.5	0.5	0.6	0.5	0.1
腎臓	1.2	1.2	1.0	1.2	1.1	0.1
肺	0.7	0.7	0.7	0.8	0.7	0.1
皮膚	0.1	0.3	0.2	0.2	0.2	0.1
骨	0.3	0.2	0.2	0.2	0.2	0.2
筋肉	0.1	0.1	0.1	0.2	0.1	0.4
尿	11.2	13.4	5.7	12.3	10.7	3.4

【0032】

表６：２４時間目の組織におけるｇ当りの注入量の％

組織	ラット#1	ラット#2	ラット#3	ラット#4	平均値	標準偏差
肝臓	0.7	0.7	0.7	0.7	0.7	0.3
脾臓	7.3	6.9	8.2	5.9	7.1	0.9
心臓	0.5	0.5	0.5	0.5	0.5	0.4
腎臓	0.6	0.6	0.5	0.6	0.6	0.6
肺	0.6	0.5	0.5	0.6	0.5	0.6
皮膚	0.1	0.1	0.1	0.1	0.1	0.1
骨	0.4	0.4	0.4	0.4	0.4	0.3
筋肉	0.1	0.1	0.1	0.1	0.1	0.2
尿 *	0.6	0.6	0.3	0.8	0.6	0.2

* １ｍＬ当りの注入量の％

【００３３】

最後に、５モル％のｍＰＥＧ$_{2000}$ －ｃ－ＤＳ又はｍＰＥＧ$_{2000}$ －ＤＳＰＥと残部のＰＨＥＰＣとを含有するリポソームを、投与後２４時間までの血液中に残留する％に関して比較した。図４に示すように、薬物動態は実際に同じであり、２４時間後に約４０％の保持であった。

【００３４】

刊行物、特許及び特許資料の全ては、個別に援用されるとしても、本明細書に援用される。種々な特定の及び好ましい実施態様を参照しながら、本発明を述べた。しかし、多くの変化及び修飾が、本発明の要旨及び範囲内に留まりながら、なされうることを理解すべきである。

【００３５】

次の実施例を例示するが、これらの実施例は如何なる意味でも本発明を限定するように意図されない。

【００３６】

実施例１Ａ．ｍＰＥＧ－ｃ－ＤＳ（ｍＰＥＧアミノプロパンジオールジステアロイル：α－メトキシ－ω－２，３－ジ（ステアロイルオキシ）プロピルカルバメートポリ（エチレンオキシド））の合成

ｍＰＥＧ$_{2000}$ （２０ｇ，１０ｍｏｌ）の溶液をトルエン（５０ｍｌ，１２０℃

）中で共沸蒸留的に乾燥させた。上記溶液の温度が２５℃に達した後に、これをニトロフェニルクロロホルメート（３．０１５ｇ，１５ｍｏｌ）によって処理し、続いてＴＥＡ（２．０１ｍｌ，１５ｍｏｌ）によって処理した。この混合物を１時間半反応させた。ＴＥＡ－塩を濾過して、溶媒を除去して、粗$mPEG_{2000}$－ニトロフェニルクロロホルメートを得て、これにアセトニトリル（５０ｍｌ）中のアミノプロパンジオール（３ｇ，３０ｍｏｌ）の溶液を加えた。この混合物を室温において一晩撹拌した。不溶物を濾過によって除去して，溶媒を蒸発させた。生成物をイソプロパノールから２回再結晶した。収量：１３．７ｇ，６５％．^{1}ＨＮＭＲ：（３００ＭＨｚ，ＤＭＳＯ－D_6）δ３．２３（ｓ，OCH_3，３Ｈ），３．６５（ｓ、ＰＥＧ，１８０Ｈ），４．０５（ｔ，ウレタンCH_2，２Ｈ），４．４２（ｔ，1° ＯＨ，１Ｈ），４．５７（ｄ，2° ＯＨ，１Ｈ）．

【００３７】

生成物、$mPEG_{2000}$ アミノプロパンジオール（２．３ｇ，１．０８ｍｏｌ，２．１７ｍｅｑのＯＨ）をトルエン（３０ｍｌ）中に溶解し、共沸蒸留的に乾燥させて、約１０ｍｌの溶液を除去した。この溶液を室温に冷却させた。ピリジン（４ｍｌ，２０％）をピペットによって加え、続いてステアロイルクロリド（１ｇ，４．３ｍｏｌ）を加えた。直ちに、白色沈殿が形成された。この反応混合物を１２０℃において一晩還流させて、冷却させた。反応フラスコの温度が約４０℃に達したときに、ピリジン塩を濾過した。濾液を蒸発させた。生成物（PEG_{2000} －ｃ－ＤＳ）をイソプロパノール（２ｘ３０ｍｌ）から２回再結晶させることによって精製して、真空下でP_2O_5上において乾燥させた。

【００３８】

収量：２．２６ｇ，８０％．ＴＬＣ（クロロホルム：メタノール，９０：１０）：mPEGアミノプロパンジオールR_f＝０．２６６；ＰＥＧ－ｃ－ＤＳ R_f＝０．５３３．

^{1}HNMR: (300 MHz, DMSO-D_6) δ 0.89 (t, CH_3, 6H), 1.26 (s, CH_2, 56 H), 1.50 (2t, $2CH_2$, 4H), 2.24 (t, $CH_2\underline{CH_2}$ C=O, 4H), 3.23 (s, OCH_3, 3H), 3.50 (s, PEG, 180H), 4.00 (dd, CH_2 of APD, 1H), 4.02 (t, CH_2OC=O-N, 2H), 4.20 (dd, CH_2 of APD, 1H), 4.98 (m, CHOC(O), 1H), 7.34 (m, NH, 1H).

【００３９】

同様な方法を利用して、分子量７５０、５０００及び１２０００のｍＰＥＧポリマーを用いてｍＰＥＧ－ｃ－ＤＳを製造した。構造をＨ－ＮＭＲと質量分析によって調べた。分子量をＭＡＬＤＩ（マトリックス介助レーザーデソープション／イオン化）によって測定して、以下に記載する：

【００４０】

コンジュゲート	MALDIによるMW
mPEG(750)-c-DS	1426
mPEG(2000)-c-DS	2892
mPEG(5000)-c-DS	5816
mPEG(12000)-c-DS	12729

【００４１】

実施例１Ｂ：ＰＥＧ－ｃ－ＤＳ（ｍＰＥＧアミノプロパンジオールジエコサノイル：α－メトキシ－ω－２，３－ジ（エコサノイルオキシ）プロピルカルバメートポリ（エチレンオキシド））の合成

１００ｍｌ丸底フラスコにおいて、エコサン酸(ecosanoic acid)（５００ｍｇ，１．６ｍｍｏｌ）をトルエン（２０ｍｌ）中に溶解し、オキサリルクロリド（１４７μｌ，１．６８ｍｍｏｌ）をピペットによって加えた。撹拌反応に、１％ＤＭＦを加えた。ＤＭＦの添加時に、ガスが放出された。このガスとの接触は全て、避けなければならない。１０分後に、トルエンを蒸発させ、追加の２０ｍｌのトルエンを加えて、蒸発させて、過剰なオキサリルクロリドを除去した。残渣を１０ｍｌのトルエン中に再溶解した。上述したように調製したｍＰＥＧ－アミノプロパンジオール（１．１９ｇ，０．５６ｍｍｏｌ）を該溶液に加え、還流冷

却管を取り付けて、混合物を一晩還流させた。ＴＬＣ（メタノールとクロロホルム，９：１）による分析は反応が完了したことを示した。反応混合物を冷却した後に、非溶解物質を濾過し、濾液を乾燥させた。生成物をイソプロパノールから３回再結晶させることによって精製し、真空下でP_2O_5上において乾燥させた。収量：１．０ｍｇ，７０％.

^{1}HNMR: (360 MHz, DMSO-D_6) δ 0.89 (t, CH_3, 6H), 1.26 (s, CH_2,脂質の66 H), 1.50 (t, $2CH_2$, 4H), 2.24 (t, $CH_2\underline{CH_2}$ C=O, 4H), 3.23 (s, OCH_3, 3H), 3.50 (s, PEG, 180H), 4.00 (dd, APDのCH_2, 1H), 4.05 (t, $CH_2\underline{CH_2}C+O$, 4H), 3.23 (s, OCH_3, 3H, 3.50 (s, PEG, 180H), 4.00 (dd, CH_2 of APD, 1H), 4.05 (t, $CH_2OC=O$–N, 2H), 4.20 (dd, CH_2 of APD, 1H), 4.98 (m, CHOC(O), 1H), 7.34 (m, NH, 1H) ppm.

【００４２】

実施例２：ｔ－Ｂｕ－Ｏ－ＰＥＧ－Ｏ－スクシンイミドを介したｔ－Ｂｕ－Ｏ－ＰＥＧ－アミノプロパンジオールの調製

Ａ．ｔ－Ｂｕ－Ｏ－ＰＥＧ－Ｏ－スクシンイミド

Ｐｏｌｙｍｅｒ　Ｌａｂｓからのｔ－Ｂｕ－Ｏ－ＰＥＧ－Ｏ－スクシンイミド（１０ｇ，５ｍｍｏｌ）を、１２０ｍｌのトルエン中に溶解して、約２０ｍｌの溶媒を除去し、Ｄｅａｎ　Ｓｔａｒｋトラップ中に水分を回収することによって、共沸蒸留的に乾燥させた。

【００４３】

該溶液を室温に冷却し、ホスゲン（１５ｍｌ）を加えた。混合物を室温において一晩反応させた。反応が完了した後に、溶媒を回転蒸発器によって除去した。約５０ｍｌの新鮮なトルエンを加えて、回転蒸発器によって除去した。残渣を乾燥トルエン（３０ｍｌ）と塩化メチレン（１０ｍｌ）中に溶解した。この溶液に、Ｎ－ヒドロキシスクシンイミド（１．７ｇ，１４．８ｍｍｏｌ）とトリエチルアミン（２．１ｍｌ，１４．９ｍｍｏｌ）とを加えて、混合物を室温において一晩反応させ、この時間後に、反応はＴＬＣによって完成していた。

【００４４】

化合物	R_f($CHCl_3$: CH_3OH, 90:10)
t-Bu-O-PEG-OH	0.44
t-Bu-O-PEG-OSc	0.51

【００４５】

塩を反応混合物から濾別し、溶媒を蒸発によって除去し、固体をイソプロピルアルコールから２回再結晶させることによって精製し、P_2O_5上で乾燥させた。収量：９．２，８５％.

over P_2O_5. Yield: 9.2, 85%. ^{1}HNMR: ($CDCl_3$, 360 MHz) δ 1.25 (s, *t*-Bu, 9H), 2.82 (s, CH_2CH_2, 4H), 3.60 (s, PEG, 180 H), 4.45 (t, CH_2OCONH, 2H) ppm.

【００４６】

B、ｔ－Ｂｕ－Ｏ－ＰＥＧ－アミノプロパンジオール

ＤＭＦ（１０ｍｌ）中のアミノプロパンジオール（３００ｍｇ，３．２ｍｍｏｌ）の溶液に、ｔ－Ｂｕ－ＰＥＧ－ＯＳｃ（５ｇ，２．２９ｍｍｏｌ）を加えて、一晩反応させた。全てのＮＨＳが消耗され、ＴＬＣ上に１スポットを示す混合物を生じた。

【００４７】

化合物	R_f($CHCl_3$: CH_3OH, 90:10)
t-Bu-O-PEG-OSc	0.51
t-Bu-O-PEG-APD	0.35

【００４８】

予め浄化した酸性イオン交換樹脂（約１ｇ）を反応混合物に加えて、３０分間後に濾過によって除去した。溶媒を除去して、残渣を２００ｍｌのイソプロピルアルコールから再結晶させた。固体を回収して、P_2O_5上で乾燥させた。収量：４．２ｇ，８５％.

^{1}HNMR: (D6-DMSO, 360 MHz) δ 1.25 (s, *t*-Bu, 9H), 3.68 (s, PEG, 180 H), 4.03 (t, CH_2OCONH, 2H), 4.43 (t, 1°OH, 1H), 4.55 (d, 2°OH, 1H), 6.98 (t, NH, 1H) ppm.

【００４９】

実施例３．ｐ－ニトロフェニルカーボネート－ＰＥＧ－ｃ－ＤＳの調製

Ａ．Ｂｎ－Ｏ－ＰＥＧ－ニトロフェニルカーボネート（ＮＰＣ）

Ｓｈｅａｒｗａｔｅｒ　Ｐｏｌｙｍｅｒｓからの Ｂｎ－Ｏ－ＰＥＧ－２０００（Ｈｕｎｔｓｖｉｌｌｅ，ＬＡ；５ｇ，２．４１ｍｍｏｌ）を、１２０ｍｌのトルエン中に溶解して、約２０ｍｌの溶媒を除去し、Ｄｅａｎ　Ｓｔａｒｋトラップ中に水を回収することによって共沸蒸留的に乾燥させた。該溶液を室温に冷却させ、残留溶媒を減圧下で蒸発させた。

【００５０】

残渣を３０ｍｌの塩化メチレン／酢酸エチル（６０：４０）中に溶解して、ｐ－ニトロフェニルクロロホルメート（７２９ｍｇ，３．６ｍｍｏｌ）とトリエチルアミン（１ｍｌ，７．２ｍｍｏｌ）とを加えた。反応を４℃において８～１６時間実施した。この方法は反応を減速させるが、ビスＰＥＧ－カーボネートの形成を排除する。ＧＦシリカプレート上のＵＶ識別可能なスポットは反応の完成を実証した。

【００５１】

反応混合物を予め浄化した酸性及び塩基性イオン交換樹脂によって３０分間処理して、濾過し、完全に乾燥させた。生成物をイソプロピルアルコールから再結晶させ、P_2O_5上で乾燥させた。収量：４．４ｇ，８０％.

【００５２】

Ｂ．Ｂｎ－Ｏ－ＰＥＧ－アミノプロパンジオール

ＤＭＦ（１０ｍｌ）中のアミノプロパンジオール（２６０ｍｇ，１．９ｍｍｏｌ）の溶液に、上記で調製したＢｎ－Ｏ－ＰＥＧ－ＮＰＣ（４．３ｇ、２．９ｍｍｏｌ）を加えて、５時間反応させた。全てのＢｎ－Ｏ－ＰＥＧ－ＮＰＣが消耗され、反応混合物はＴＬＣ（クロロホルム：メタノール：水　９０：１８：２）

上に１スポットを生じた。

【００５３】

この反応混合物を５ｇの予め浄化した酸性イオン交換樹脂によって３０分間処理し、濾過し、完全に乾燥させた。生成物をイソプロピルアルコールから再結晶させ、P_2O_5上で乾燥させた。収量：３．８ｇ，９１％．

【００５４】

Ｃ．Ｂｎ−Ｏ−ＰＥＧ−ｃ−ジステアロイル

Ｂｎ−Ｏ−ＰＥＧ−アミノプロパンジオール（３ｇ，１．３６ｍｍｏｌ）と、ステアリン酸（１．９４ｇ，６．７９ｍｍｏｌ）と、触媒としてのＤＰＴＳ（４−ジメチルアミノ）ピリジニウム４−トルエンスルホネート）（４０８ｍｇ，１．３６ｍｍｏｌ）との溶液を室温において２０分間撹拌した。ジイソプロピルカルボジイミド（１．２８ｍｌ，８．１６ｍｍｏｌ）をピペットによって加え、混合物を一晩反応させた。ＴＬＣ（クロロホルム：メタノール，９０：１０）はジオールの完全な反応を示した。

【００５５】

塩基性イオン交換樹脂（約５ｇ）を反応混合物に加えた。３０分間振とうした後に、樹脂を濾過し、濾液を乾燥させた。残渣をイソプロピルアルコール（１００ｍｌ）から再結晶させ、P_2O_5上で乾燥させた。収量：４ｇ，８０％．

【００５６】

Ｄ．ＨＯ−ＰＥＧ−ｃ−ジステアロイル

Ｂｎ−Ｏ−ＰＥＧ−ｃ−ＤＳのベンジル基を脱保護するために、２種類の異なるアプローチを採用した。

【００５７】

方法１．水素化分解：炭素付きパラジウムによる脱保護．　５ｍｌのメタノール中のＢｎ−Ｏ−ＰＥＧ−ｃ−ＤＳ（２１８ｍｇ，０．０８ｍｍｏｌ）の溶液に、１０％Ｐｄ／Ｃ（１１０ｍｇ）とギ酸アンモニウム（１０７ｍｇ，０．８ｍｍｏｌ）とを加えて、混合物を室温において一晩反応させた。Ｐｄ／ＣをＣｅｌｉｔｅ(登録商標)上で濾過によって除去して、濾液を乾燥させた。残渣をクロロホルム中に溶解して、飽和ＮａＣｌによって３回洗浄した。クロロホルム相を回収

し、$MgSO_4$によって乾燥させ、濾過して、濃縮した。固体残渣をｔＢｕＯＨから凍結乾燥させ、得られた粉末をP_2O_5上で乾燥させた。収量：８０％，１７５ｍｇ．

【００５８】

方法２：四塩化チタンによる脱保護．　塩化メチレン（１０ｍｌ）中のＢｎ－Ｏ－ＰＥＧ－ｃ－ＤＳ（１．１８ｇ，０．４３ｍｍｏｌ）の溶液を氷浴中で１５分間冷却した。四塩化チタン（３ｍｌ，２１．５ｍｏｌ，過剰量）をオーブン乾燥シリンジによって密封反応フラスコに移した。５分間後に、氷浴を除去し、脱保護反応を室温において一晩実施した。ＧＦシリカＴＬＣプレート上の下方にシフトしたスポット（出発物質に比べて）によって完全な脱保護が示された。

【００５９】

約４０ｍｌのクロロホルムを反応混合物に加えて、混合物を４０ｍｌの飽和$NaHCO_3$を含有する分液ロートに移した。混合物を穏やかに振とうして（エマルジョンの形成を避けるために）、クロロホルム層を回収した。この抽出を３回繰り返し、クロロホルム相を回収して、$TiCl_4$の完全な除去を保証するために飽和$NaHCO_3$の新たな部によってもう１回抽出した。回収したクロロホルム相を$MgSO_4$によって乾燥させ、濾過し、濃縮した。

【００６０】

上記残渣を１ｍｌのクロロホルム中に溶解して、シリカゲル（２００－４００メッシュ，６０Å）の準備されたカラムに加えた。移動相(クロロホルム）の極性は、生成物が１０％メタノール／９０％クロロホルムにおいて溶出するまで、メタノールを２％増分で添加することによって上昇した。生成物を回収し、溶媒を回転蒸発器によって除去した。固体をｔＢｕＯＨから凍結乾燥し、P_2O_5上で乾燥させた。収量：７０％，８００ｍｇ．

【００６１】

Ｅ．ｐ－ニトロフェニルカーボネート－ＰＥＧ－ｃ－ＤＳ

反応の開始前に、反応フラスコ、撹拌バー、シリンジ及び出発物質（ＨＯ－ＰＥＧ－ｃ－ＤＳ，上記で調製）を細心に乾燥させた。

【００６２】

１０ｍｌの塩化メチレン／酢酸エチル（６０：４０）中のＨＯ－ＰＥＧ－ｃ－ＤＳ（１．２ｇ，０．４５ｍｍｏｌ）の溶液に、ｐ－ニトロフェニルカーボネート（１３６ｍｇ，０．６５ｍｍｏｌ）とトリエチルアミン（１８８μｌ，１．３５ｍｍｏｌ）とを加えた。反応を４℃において８～１６時間実施し（ビスＰＥＧ－カーボネートの形成を排除するために）、この時間後に、反応はＧＦシリカゲルＴＬＣによって完成していた。

【００６３】

化合物	R_f ($CHCl_3$: CH_3OH, 90:10)
HO-PEG-c-DS	0.40
NPC-PEG-c-DS	0.54

【００６４】

反応混合物を予め浄化した酸性及び塩基性イオン交換樹脂によって３０分間処理して、濾過した。濾液を完全に乾燥させ、残渣をイソプロピルアルコールから再結晶させた。固体をP_2O_5上で乾燥させた。収量：７０％.

^{1}NHMR: (D6-DMSO, 360 MHz) δ 0.86 (t, CH_3, 6 H), 1.22 (s, DS, 56H), 1.48 (m, CH_2CH_2(CO)), 4H), 2.26 (2 xt, CH_2OCONH, 2H), 4.03 & 4.22 (2 xd,脂質のCH_2CH, 2H), 4.97 (M,脂質の$CH$$CH_2$), 6.98 (t, NH, 1H), 7.55 %8.32 (2xd,芳香族, 4H) ppm.

【００６５】

実施例４：ＰＥＧ－ＤＳＰＥ－及びＰＥＧ－ｃ－ＤＳ－含有リポソームの調製と生体分布研究

下記比率でのＨＳＰＣ：Ｃｈｏｌ：ＰＥＧ脂質の混合物から溶解と溶媒の除去によって、脂質フィルムを形成した：

A: 58:39:3; 脂質 = PEG-c-DS

B: 57:38:5; 脂質 = PEG-DSPE

C: 57:38:5; 脂質 = PEG-c-DS

【0066】

140mM NaCl、pH7．4を含有する25mM HEPES中の新たに調製された^{125}I-チラミニルイヌリン中で該フィルムを水和し、押し出して、直径100～105nmのリポソームを形成した。これらのリポソームを0．22μmのMillipore（Millipore Corporation，Bedford，MA）低タンパク質結合シリンジ末端フィルターを通しての濾過によって滅菌した。アリコートをカウントして、^{125}Iの注入カウントを調べた。リポソーム調製物のホスフェート含量を分析することによって、脂質濃度を測定し、リポソーム調製物を無菌緩衝液中で2．5μmol／mlの最終濃度に希釈した。マウスに尾静脈から、各マウスが0．5μmolのリン脂質を受容するように、0．2mlの希釈リポソームを注入した。種々な時点において、マウスをハロタン麻酔とその後の頸部脱臼とによって安楽死させ、血液を心臓出血によってサンプリングし、血液と種々な器官とを^{125}Iカウントに関して分析した。

【図面の簡単な説明】

【図1】

図1は、カルバメート連結非荷電リポポリマー（本明細書では、PEG-c-DSと呼ぶ）の調製の合成スキームを示す。

【図2】

図2A～2Dは、エーテル-、エステル-、アミド-及びケト-連結非荷電リポポリマーの調製の合成スキームを示す。

【図3】

図3A～3Cは、血液、肝臓及び脾臓中の3モル%PEG-c-DS（A）；5モル%PEG-DSPE（B）；又は5モル%PEG-c-DS（C）を含有するHSPC／Cholリポソームの生体分布を示すグラフである。

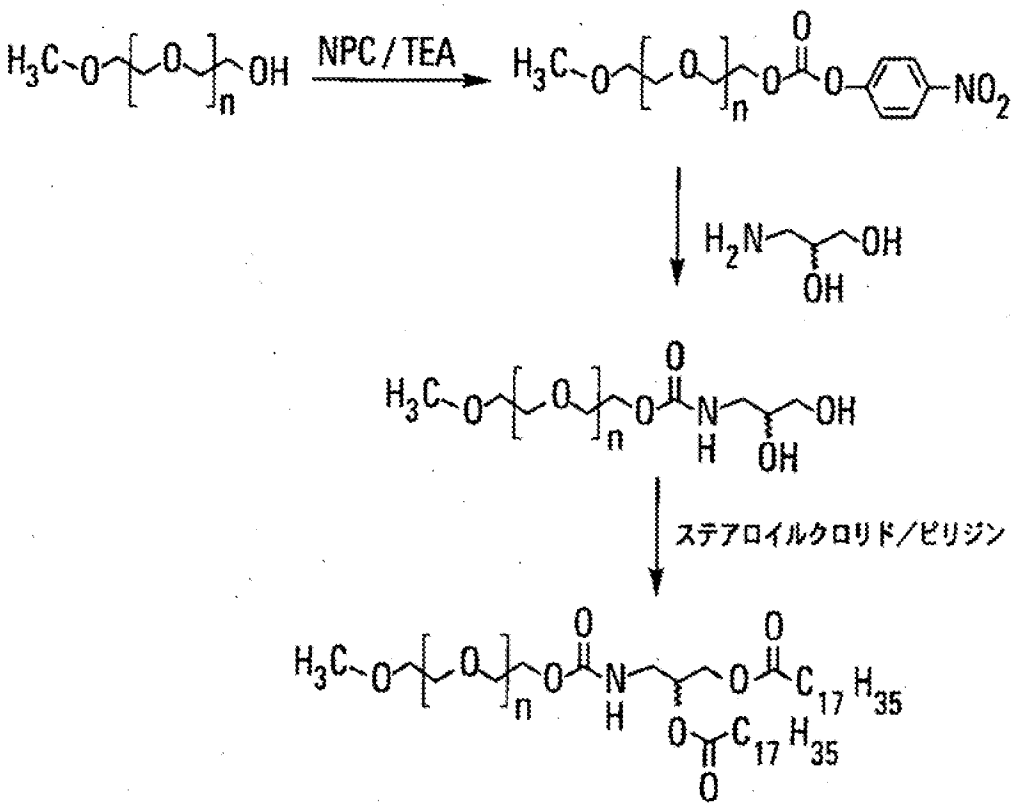
【図4】

図4は、2：1HSPC PEGフリーリポソーム（即ち、PEG含まず）（クロス）、5モル%PEG-DSPE（三角）及び5モル%PEG-c-DS（円）の血液中の保持を示すグラフである。

【図５】

図５は、５モル％ＰＥＧ－ｃ－ＤＳ（円）と５モル％ＰＥＧ－ＤＳＰＥ（四角）を含有するＰＨＥＰＣリポソームの血液中の保持を示すグラフである。

【図１】



【図２Ａ】

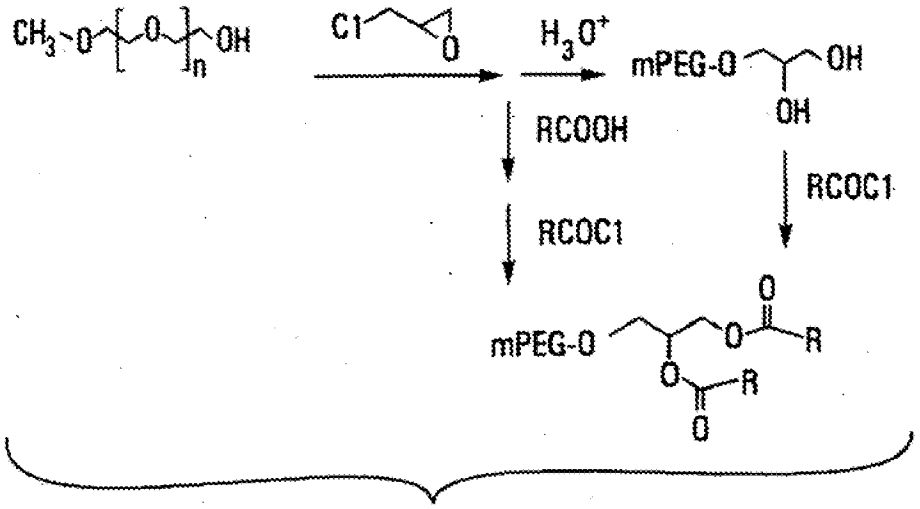


FIG. 2A

【図２Ｂ】

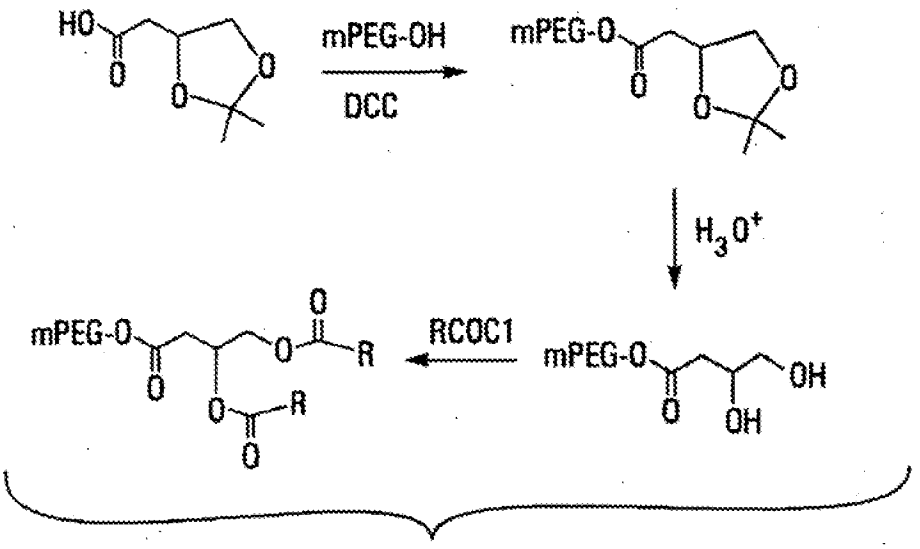


FIG. 2B

【図２Ｃ】

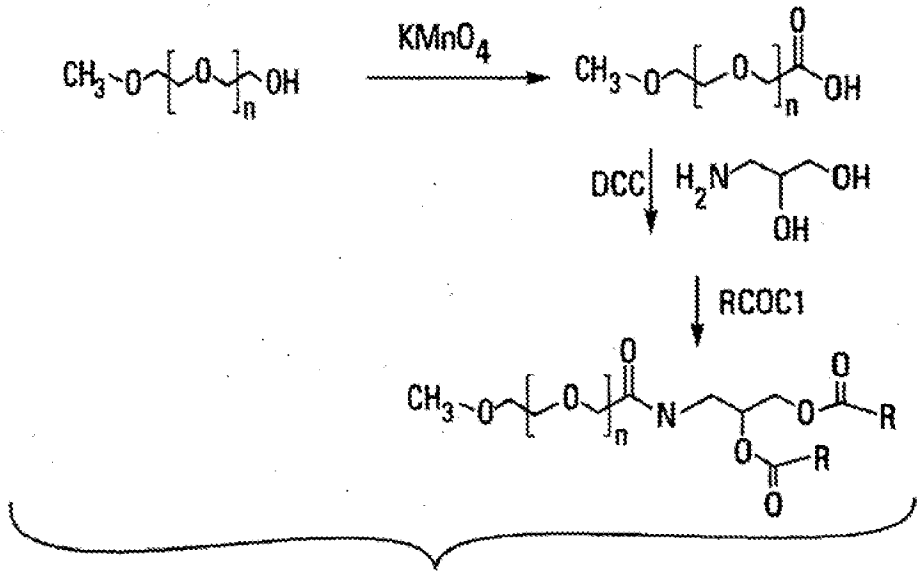


FIG. 2C

【図２Ｄ】

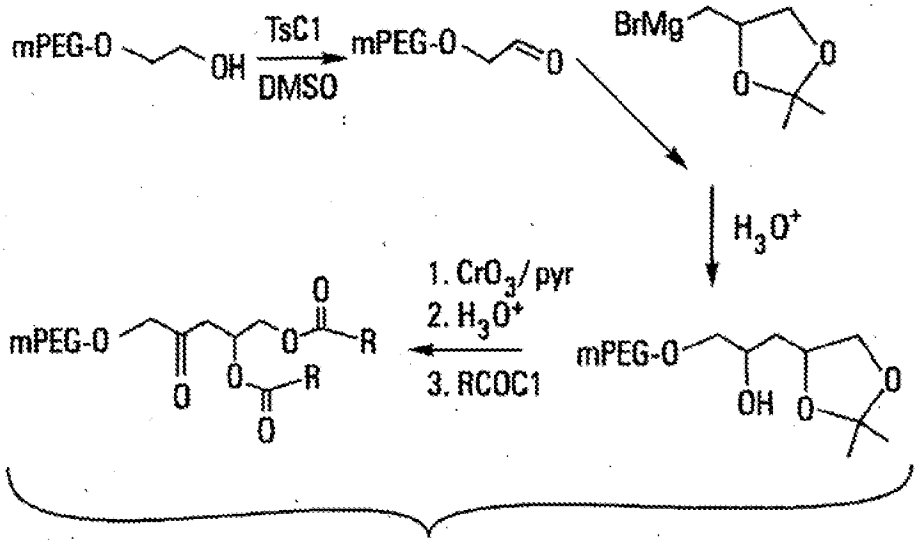
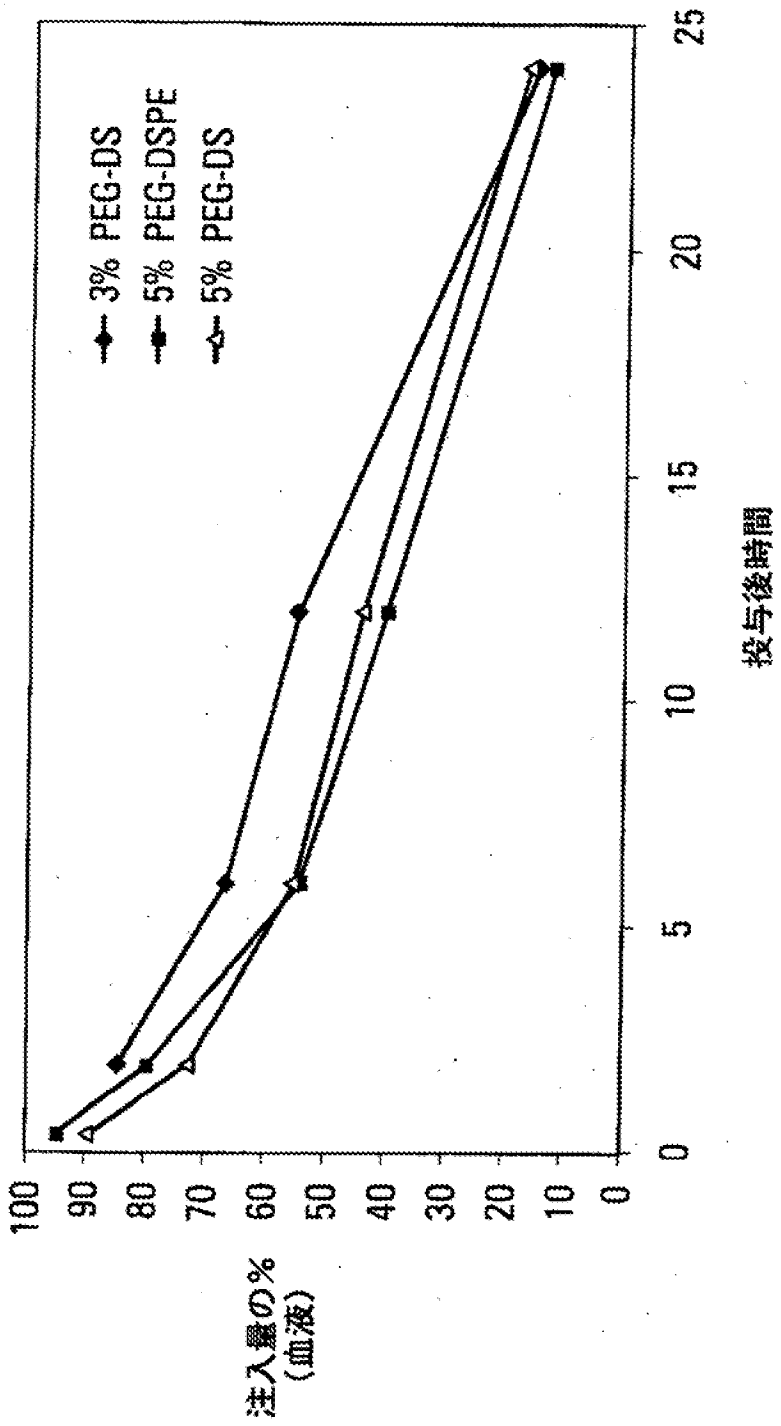
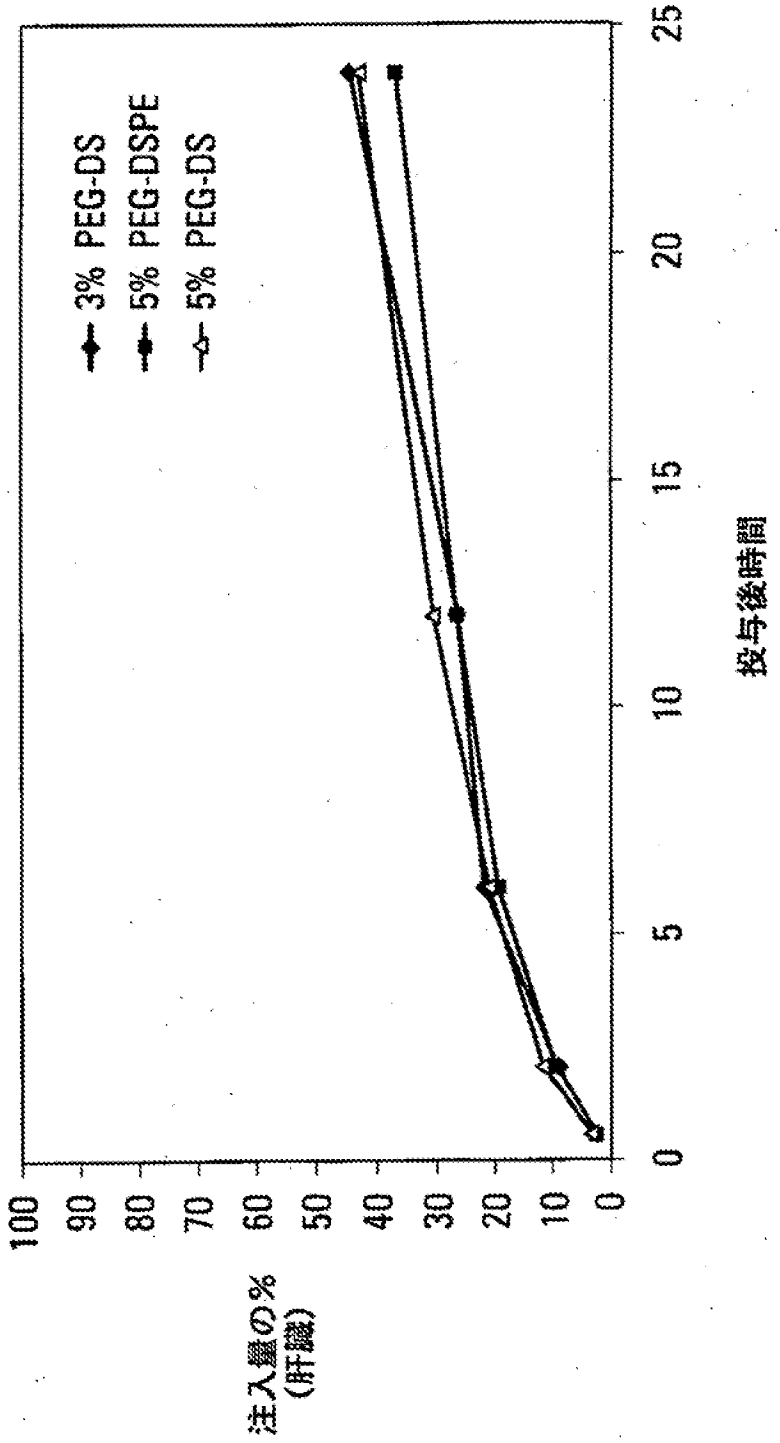


FIG. 2D

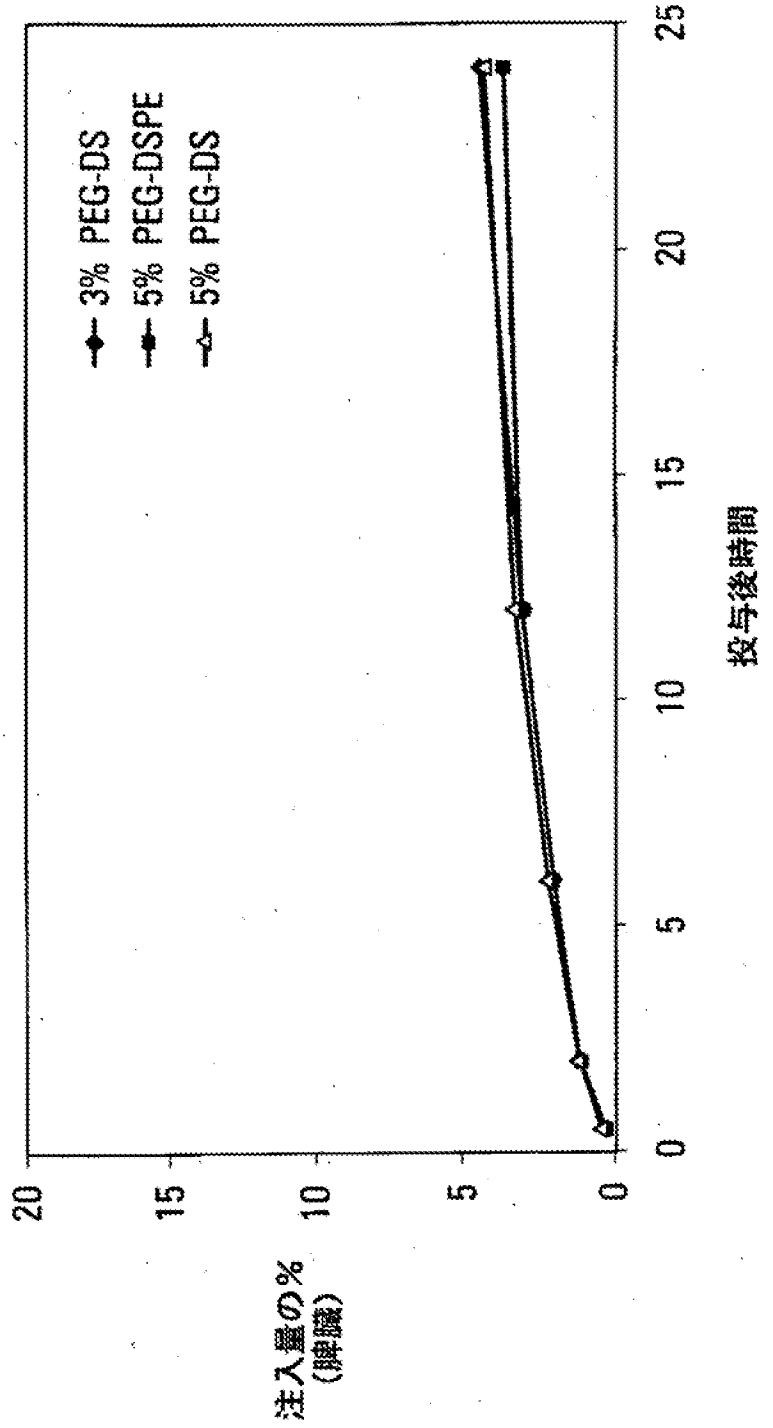
【図３Ａ】


3% PEG-DS
5% PEG-DSPE
5% PEG-DS
注入量の%
(血液)
投与後時間
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25

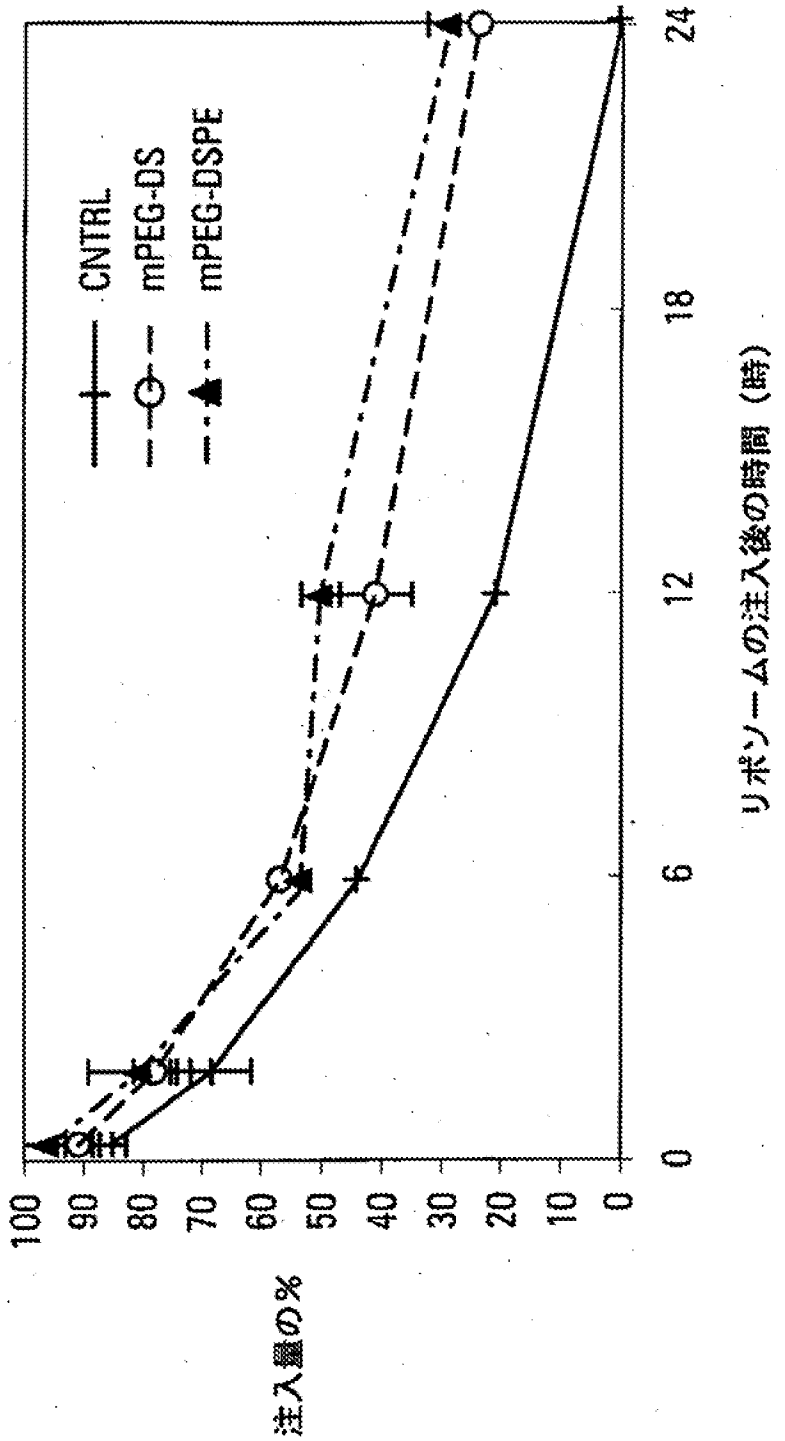
【図３Ｂ】


3% PEG-DS
5% PEG-DSPE
5% PEG-DS
注入量の%
(肝臓)
投与後時間
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25

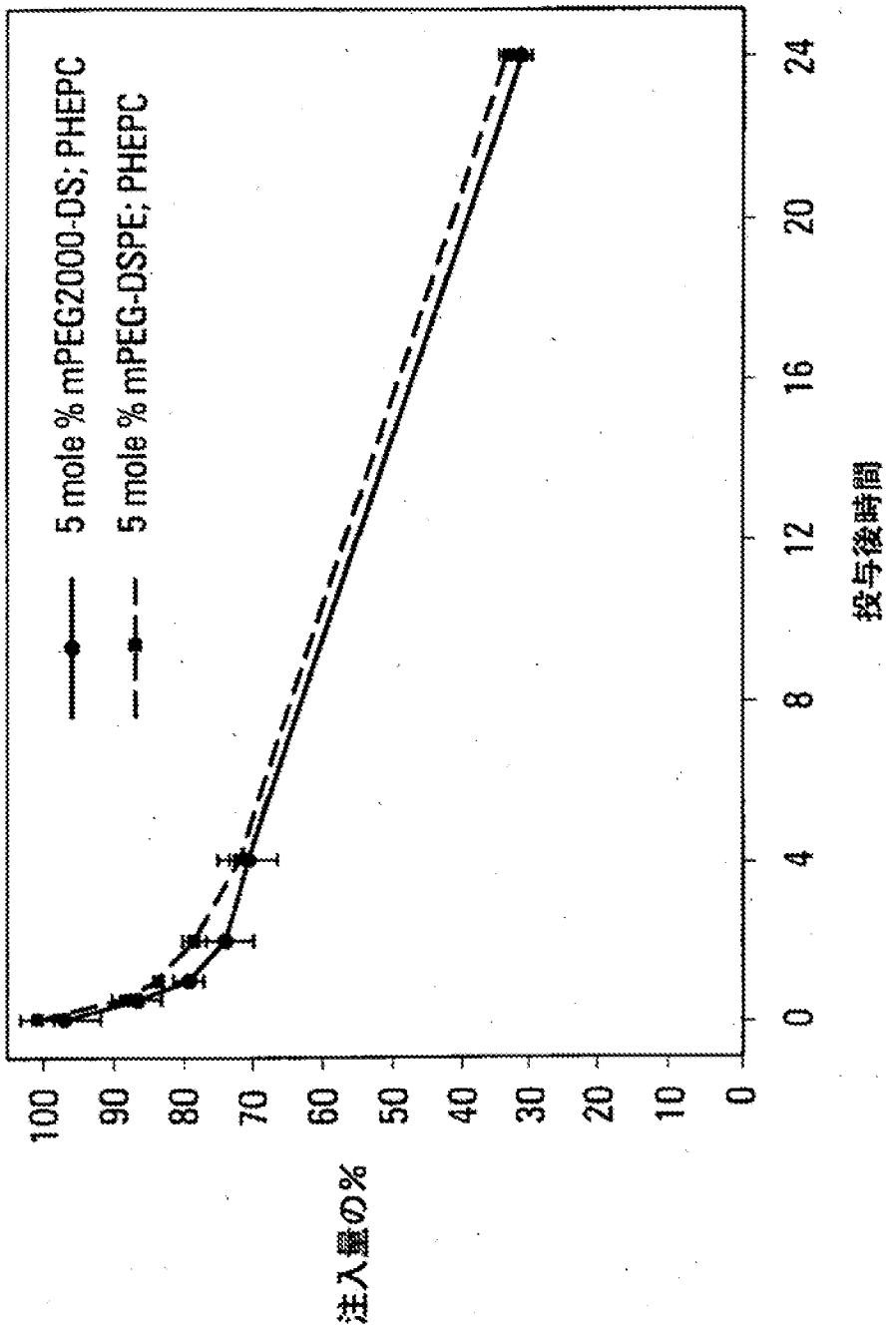
【図３Ｃ】



【図４】


CNTRL
mPEG-DS
mPEG-DSPE
注入量の％
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
リポソームの注入後の時間（時）

【図５】


5 mole % mPEG2000-DS; PHEPC
5 mole % mPEG-DSPE; PHEPC
注入量の%
投与後時間
0
10
20
30
40
50
60
70
80
90
100
0
4
8
12
16
20
24

【手続補正書】特許協力条約第３４条補正の翻訳文提出書

【提出日】平成１３年１０月２６日（２００１．１０．２６）

【手続補正１】

【補正対象書類名】明細書

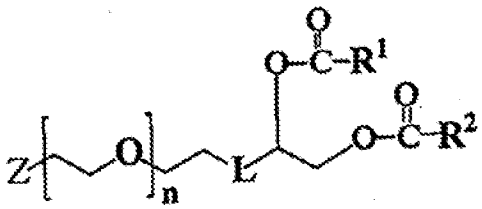
【補正対象項目名】特許請求の範囲

【補正方法】変更

【補正の内容】

【特許請求の範囲】

【請求項１】　式：



［式中、R^1とR^2の各々は約８～約２４個の炭素原子を有するアルキル又はアルケニル鎖であり；ｎ＝約１０～約３００；Ｚはヒドロキシ、アルコキシ、ベンジルオキシ、カルボン酸エステル、スルホン酸エステル、アルキル又はアリールカーボネート、アミノ及びアルキルアミノから成る群から選択され；Ｌは（ｉ）－Ｘ－（Ｃ＝Ｏ）－Ｙ－CH_2－、（ｉｉ）－Ｘ－（Ｃ＝Ｏ）－及び（ｉｉｉ）－Ｘ－CH_2－から成る群から選択され、この場合、ＸとＹは酸素、ＮＨ及び直接結合から独立的に選択される；但し、Ｌが－Ｘ－（Ｃ＝Ｏ）－である場合には、ＸはＮＨではない］で示される中性リポポリマー約１モル％～約１０モル％と、残部のベシクル形成脂質とを含むリポソーム組成物。

【請求項２】　Ｘが酸素であり、Ｙが窒素である、請求項１記載の組成物。

【請求項３】　Ｌがカルバメート結合、エステル結合又はカーボネート結合である、請求項１記載の組成物。

【請求項４】　Ｌが－Ｏ－（Ｃ＝Ｏ）－ＮＨ－CH_2－（カルバメート結合）である、請求項３記載の組成物。

【請求項５】　Ｚがヒドロキシ又はメトキシである、請求項１記載の組成物

。

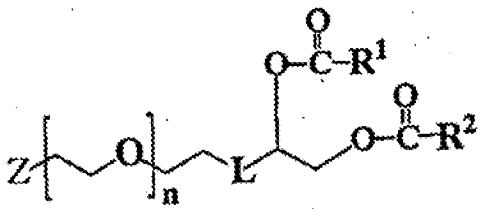
【請求項６】　約３モル％～約６モル％の中性リポポリマーをさらに含む、請求項１記載の組成物。

【請求項７】　R^1とR^2の各々が約８～約２４個の炭素原子を有する非分枝アルキル又はアルケニル鎖である、請求項１記載の組成物。

【請求項８】　R^1とR^2の各々が$C_{17}H_{35}$である、請求項６記載の組成物。

【請求項９】　ｎが約２０～約１１５である、請求項１記載の組成物。

【請求項１０】　ベシクル形成脂質を含むリポソームの循環時間を延長する方法であって、前記リポソーム中にベシクル形成脂質と共に、式：



［式中、R^1とR^2の各々は約８～約２４個の炭素原子を有するアルキル又はアルケニル鎖であり；ｎ＝約１０～約３００；Ｚはヒドロキシ、アルコキシ、ベンジルオキシ、カルボン酸エステル、スルホン酸エステル、アルキル又はアリールカーボネート、アミノ及びアルキルアミノから成る群から選択され；Ｌは（ｉ）－Ｘ－（Ｃ＝Ｏ）－Ｙ－CH_2－、（ｉｉ）－Ｘ－（Ｃ＝Ｏ）－及び（ｉｉｉ）－Ｘ－CH_2－から成る群から選択され、この場合、ＸとＹは酸素、ＮＨ及び直接結合から独立的に選択される、但し、Ｌが－Ｘ－（Ｃ＝Ｏ）－である場合には、ＸはＮＨではない］で示される中性リポポリマー　約１モル％～約１０モル％を組み込むことを含む前記方法。

【請求項１１】　Ｘが酸素であり、Ｙが窒素である、請求項１０記載の方法

。

【請求項１２】　Ｌがカルバメート結合、エステル結合又はカーボネート結合である、請求項１０記載の方法。

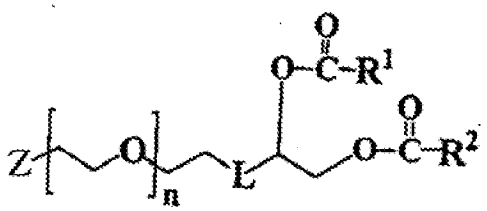
【請求項１３】　Ｌが－Ｏ－（Ｃ＝Ｏ）－ＮＨ－CH_2－（カルバメート結合）である、請求項１２記載の方法。

【請求項１４】　Ｚがヒドロキシ又はメトキシである、請求項１０記載の方法。

【請求項１５】　約３モル％～約６モル％の中性リポポリマーを組み込む、請求項１０記載の方法。

【請求項１６】　ｎが約２０～約１１５である、請求項１０記載の方法。

【請求項１７】　式：



［式中、R^1とR^2の各々は約８～約２４個の炭素原子を有するアルキル又はアルケニル鎖であり；ｎ＝約１０～約３００；Ｚはヒドロキシ、アルコキシ、ベンジルオキシ、カルボン酸エステル、スルホン酸エステル、アルキル又はアリールカーボネート、アミノ及びアルキルアミノから成る群から選択され；Ｌは（ｉ）－Ｘ－（Ｃ＝Ｏ）－Ｙ－CH_2－、（ｉｉ）－Ｘ－（Ｃ＝Ｏ）－及び（ｉｉｉ）－Ｘ－CH_2－から成る群から選択され、この場合、ＸとＹは酸素、ＮＨ及び直接結合から独立的に選択される、但し、Ｌが－Ｘ－（Ｃ＝Ｏ）－である場合には、ＸはＮＨではない］で示される中性リポポリマー。

【請求項１８】　Ｘが酸素であり、Ｙが窒素である、請求項１７記載のリポポリマー。

【請求項１９】　Ｌがカルバメート結合、エステル結合又はカーボネート結合である、請求項１７記載のリポポリマー。

【請求項２０】　Ｌが－Ｏ－（Ｃ＝Ｏ）－ＮＨ－CH_2－（カルバメート結合）である、請求項１９記載のリポポリマー。

【請求項２１】　R^1とR^2の各々が約８～約２４個の炭素原子を有する非分枝アルキル又はアルケニル鎖である、請求項１７記載のリポポリマー。

【請求項２２】　R^1とR^2の各々が$C_{17}H_{35}$である、請求項２１記載のリポポリマー。

【請求項２３】　Ｚがヒドロキシ又はメトキシである、請求項１７記載のリポポリマー。

【請求項２４】　ｎが約２０～約１１５である、請求項１７記載のリポポリマー。

【手続補正２】

【補正対象書類名】明細書

【補正対象項目名】０００７

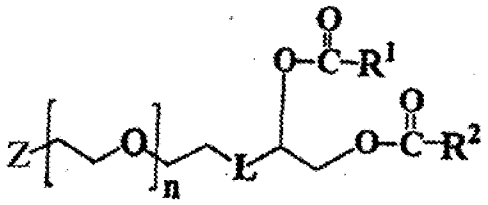
【補正方法】変更

【補正の内容】

【０００７】

（発明の概要）

１態様において、本発明は、約１モル％～約１０モル％の中性リポポリマーを有するリポソーム組成物であって、該中性リポポリマーが式：



［式中、R^1とR^2の各々は約８～約２４個の炭素原子を有するアルキル又はアルケニル鎖であり；ｎ＝約１０～約３００；Ｚはヒドロキシ、アルコキシ、ベンジルオキシ、カルボン酸エステル、スルホン酸エステル、アルキル又はアリールカーボネート、アミノ及びアルキルアミノから成る群から選択され；Ｌは（ｉ）－Ｘ－（Ｃ＝Ｏ）－Ｙ－CH_2－、（ｉｉ）－Ｘ－（Ｃ＝Ｏ）－及び（ｉｉｉ）－Ｘ－CH_2－から成る群から選択され、この場合、ＸとＹは酸素、ＮＨ及び直接結合から独立的に選択される］によって示される前記リポソーム組成物を包含する。好ましくは、該組成物は約３モル％～約６モル％の中性リポポリマーを包含する。

【手続補正３】

【補正対象書類名】明細書

【補正対象項目名】００１５

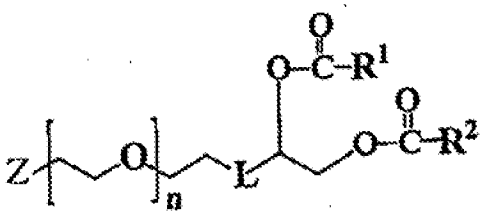
【補正方法】変更

【補正の内容】

【００１５】

ＩＩ．中性リポポリマー

本発明のＰＥＧ置換中性リポポリマーは以下に示す構造を有する：



式中、R^1とR^2の各々は約８～約２４個の炭素原子を有するアルキル又はアルケニル鎖であり；ｎは約１０～約３００であり；Ｚはヒドロキシ、アルコキシ、ベンジルオキシ、カルボン酸エステル、スルホン酸エステル、アルキル又はアリールカーボネート、アミノ及びアルキルアミノから成る群から選択され；Ｌは（ｉ）－Ｘ－（Ｃ＝Ｏ）－Ｙ－CH_2－、（ｉｉ）－Ｘ－（Ｃ＝Ｏ）－及び（ｉｉｉ）－Ｘ－CH_2－から成る群から選択され、この場合、ＸとＹは酸素、ＮＨ及び直接結合から独立的に選択される。

【国際調査報告】

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 00/18949

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C08G65/329 A61K9/127

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 7 C08G A61K

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)
EPO-Internal, CHEM ABS Data, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 786 387 A (ANDO TAKASHI ET AL) 28 July 1998 (1998-07-28) example 5 claims 1,8,14,15	1,5-7,9, 10, 14-17, 21,23,26
A	US 5 891 468 A (ZALIPSKY SAMUEL ET AL) 6 April 1999 (1999-04-06) cited in the application figure 8	1-24

-/--

[X] Further documents are listed in the continuation of box C. [X] 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

Date of the actual completion of the international search	Date of mailing of the international search report
31 October 2000	15 11 2000
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 O'Sullivan, T

Form PCT/ISA/210 (second sheet) (July 1998)

page 1 of 2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/18949

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 18480 A (NEXSTAR PHARMACEUTICALS INC ;SOLD LARRY (US); JANJIC NEBOJSA (US);) 7 May 1998 (1998-05-07) Compound no. 20 page 48 compound no. 8 page 68	1-24
A	FR 2 694 893 A (OREAL) 25 February 1994 (1994-02-25) claim 1	1-24
A	WO 96 10391 A (UNIV BRITISH COLUMBIA) 11 April 1996 (1996-04-11) claims 1-30	1-24

2

Form PCT/ISA/210 (continuation of second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/18949

Box I Observations where certain claims were found unsearchable (Continuation of item 1 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. [X] Claims Nos.: 1-24 (ALLIN PART)
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:
see FURTHER INFORMATION sheet PCT/ISA/210

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 II Observations where unity of invention is lacking (Continuation of item 2 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.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 00 18949

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-24 (ALLIN PART)

Present claims 1-24 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of said compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds as disclosed in the present examples. Note in particular with respect to present claims 1, 10 and 14 that the search has been limited to the ester derivatives disclosed in the present examples rather than the ether derivatives as defined in the claims, as no support for the preparation of the ether derivatives can be found in the application as filed.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inter... nal Application No
PCT/US 00/18949

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
US 5786387	A	28-07-1998	WO	9529764	A	28-09-1995
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			EP	0957929	A	24-11-1999
FR 2694893	A	25-02-1994	AT	145544	T	15-12-1996
			AU	4434193	A	10-02-1994
			CA	2101760	A	04-02-1994
			DE	69306190	D	09-01-1997
			DE	69306190	T	03-04-1997
			DK	582503	T	24-02-1997
			EP	0582503	A	09-02-1994
			ES	2095015	T	01-02-1997
			FR	2694884	A	25-02-1994
			GR	3022513	T	31-05-1997
			JP	2772751	B	09-07-1998
			JP	6184002	A	05-07-1994
			US	5741518	A	21-04-1998
			US	5866158	A	02-02-1999
			ZA	9305568	A	01-03-1994
WO 9618391	A	11-04-1996	AT	177943	T	15-04-1999
			AU	3559895	A	26-04-1996
			CA	2201120	A	11-04-1996
			DE	69508598	D	29-04-1999
			DE	69508598	T	15-07-1999
			DK	783297	T	11-10-1999
			EP	0783297	A	16-07-1997
			ES	2130651	T	01-07-1999
			JP	10506622	T	30-06-1998
			US	5820873	A	13-10-1998

Form PCT/ISA/210 (patent family annex) (July 1992)

フロントページの続き

(51)Int.Cl.[7]	識別記号	F I	ﾃｰﾏｺｰﾄﾞ(参考)
//(C08L 91/00 71:02)		C08L 71:02	

(81)指定国 EP(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OA(BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG), AP(GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), EA(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, 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, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW

(19)日本国特許庁(JP)　(12)公表特許公報(A)　(11)特許出願公表番号
特表2007-524349
(P2007-524349A)
(43)公表日　平成19年8月30日(2007.8.30)

(51)Int.Cl.		F I		テーマコード（参考）
C12N 15/09	(2006.01)	C12N 15/00	ZNAA	4B024
C12N 5/10	(2006.01)	C12N 5/00	B	4B065
A61K 31/713	(2006.01)	A61K 31/713		4C076
A61K 35/76	(2006.01)	A61K 35/76		4C084
A61K 48/00	(2006.01)	A61K 48/00		4C086

審査請求 有　予備審査請求 有　（全 37 頁）　最終頁に続く

(21)出願番号　特願2006-500988 (P2006-500988)
(86)(22)出願日　平成16年1月16日 (2004.1.16)
(85)翻訳文提出日　平成17年9月16日 (2005.9.16)
(86)国際出願番号　PCT/US2004/001166
(87)国際公開番号　WO2004/065546
(87)国際公開日　平成16年8月5日 (2004.8.5)
(31)優先権主張番号　60/440,579
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(33)優先権主張国　米国 (US)

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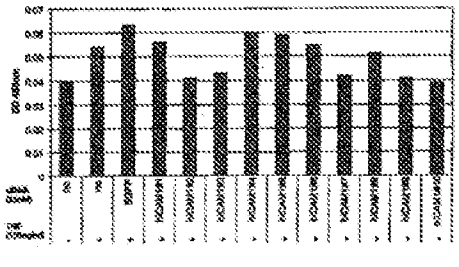
最終頁に続く

(54)【発明の名称】ＩＣＡＭ－１のｓｉＲＮＡ阻害のための組成物及び方法

(57)【要約】
【解決手段】　ＩＣＡＭ－１遺伝子に特異的な低分子干渉ＲＮＡを用いたＲＮＡ干渉は、この遺伝子の発現を阻害する。ＩＣＡＭ－１仲介性細胞接着を伴う疾患、例えば、炎症性疾患及び自己免疫疾患、糖尿病性網膜症及び糖尿病Ｉ型から生じる他の合併症、年齢関連性黄斑変性症、及び様々なタイプの癌などを、前記低分子干渉ＲＮＡを投与することによって治療され得るものである。
【選択図】　図２



【特許請求の範囲】
【請求項1】
単離されたｓｉＲＮＡであって、
センスＲＮＡ鎖及びアンチセンスＲＮＡ鎖を有し、前記センスＲＮＡ鎖及びアンチセンスＲＮＡ鎖はＲＮＡ二本鎖を形成するものであり、
前記センスＲＮＡ鎖は、ヒトＩＣＡＭ－１　ｍＲＮＡ（配列ＩＤ番号：１）における約１９～約２５の近接するヌクレオチドの標的配列と実質的に同一であるヌクレオチド配列、若しくは、それらの選択的スプライシング体、変異体、或いは同族体を有するものである、ｓｉＲＮＡ。
【請求項2】
請求項１のｓｉＲＮＡにおいて、
前記ヒトＩＣＡＭ－１　ｍＲＮＡ配列の同族体は、マウスＩＣＡＭ－１　ｍＲＮＡ（配列ＩＤ番号：２）である。
【請求項3】
請求項１のｓｉＲＮＡにおいて、
前記センスＲＮＡ鎖は１つのＲＮＡ分子を有し、前記アンチセンスＲＮＡ鎖は１つのＲＮＡ分子を有するものである。
【請求項4】
請求項１のｓｉＲＮＡにおいて、
前記ＲＮＡ二本鎖を形成する前記センスＲＮＡ鎖及びアンチセンスＲＮＡ鎖は、一本鎖ヘアピンによって共有結合的に連結されているものである。
【請求項5】
請求項１のｓｉＲＮＡにおいて、
前記ｓｉＲＮＡはさらに、非ヌクレオチド物質を有するものである。
【請求項6】
請求項１のｓｉＲＮＡにおいて、
前記ｓｉＲＮＡはさらに、１若しくはそれ以上のヌクレオチドの付加、欠損、置換、若しくは変化を有するものである。
【請求項7】
請求項１のｓｉＲＮＡにおいて、
前記センスＲＮＡ鎖及びアンチセンスＲＮＡ鎖は、ヌクレアーゼ分解に対して安定化されているものである。
【請求項8】
請求項１のｓｉＲＮＡであって、このｓｉＲＮＡは、さらに、
３’オーバーハングを有するものである。
【請求項9】
請求項８のｓｉＲＮＡにおいて、
前記３’オーバーハングは、１～約６ヌクレオチドを有するものである。
【請求項10】
請求項８のｓｉＲＮＡにおいて、
前記３’オーバーハングは、約２ヌクレオチドを有するものである。
【請求項11】
請求項３のｓｉＲＮＡにおいて、
前記センスＲＮＡ鎖は、第１の３’オーバーハングを有し、前記アンチセンスＲＮＡ鎖は、第２の３’オーバーハングを有するものである。
【請求項12】
請求項１１のｓｉＲＮＡにおいて、
前記第１及び第２の３’オーバーハングは、別々に１～約６ヌクレオチドを有するものである。

【請求項13】
請求項１２のｓｉＲＮＡにおいて、
前記第１の３’オーバーハングはジヌクレオチドを有し、第２の３’オーバーハングはジヌクレオチドを有するものである。
【請求項14】
請求項１３のｓｉＲＮＡにおいて、
前記第１と第２の３’オーバーハングを有する前記ジヌクレオチドは、ジチミジル酸（ＴＴ）若しくはジウリジル酸（ｕｕ）である。
【請求項15】
請求項８のｓｉＲＮＡにおいて、
前記３’オーバーハングは、ヌクレアーゼ分解に対して安定化されているものである。
【請求項16】
請求項１のｓｉＲＮＡを有する、網膜内皮細胞。
【請求項17】
センスＲＮＡ鎖とアンチセンスＲＮＡ鎖を有するｓｉＲＮＡを発現するための核酸配列を有する組換えプラスミドであって、
前記センスＲＮＡ鎖及びアンチセンスＲＮＡ鎖は、ＲＮＡ二本鎖を形成しており、前記センスＲＮＡ鎖は、ヒトＩＣＡＭ－１　ｍＲＮＡにおける約１９～約２５の隣接したヌクレオチドの標的配列と実質的に同一なヌクレオチド配列、若しくはそれらの選択的スプライシング体、変異体、或いは同族体を有する、組換えプラスミド。
【請求項18】
請求項１７の組換えプラスミドにおいて、
前記ｓｉＲＮＡを発現するための核酸配列は、誘導性若しくは制御性プロモーターを有するものである。
【請求項19】
請求項１７の組換えプラスミドにおいて、
前記ｓｉＲＮＡを発現するための核酸配列は、ヒトＵ６ＲＮＡプロモーターの制御下でのｐｏｌｙＴ終止配列との操作可能な連結を持つ配列をコード化するセンスＲＮＡ鎖と、ヒトＵ６　ＲＮＡプロモーターの制御下でのｐｏｌｙＴ終止配列との操作可能な連結を持つ配列をコード化するアンチセンスＲＮＡ鎖とを有するものである。
【請求項20】
請求項１７の組換えベクターにおいて、
前記プラスミドは、ＣＭＶプロモーターを有するものである。
【請求項21】
センスＲＮＡ鎖とアンチセンスＲＮＡ鎖を有するｓｉＲＮＡを発現するための核酸配列を有する組換えウイルスベクターであって、
前記センスＲＮＡ鎖及びアンチセンスＲＮＡ鎖は、ＲＮＡ二本鎖を形成しており、前記センスＲＮＡ鎖は、ヒトＩＣＡＭ－１ｍＲＮＡにおける約１９～約２５の隣接したヌクレオチドの標的配列と実質的に同一なヌクレオチド配列、若しくはそれらの選択的スプライシング体、変異体、或いは同族体を有する、組換えウイルスベクター。
【請求項22】
請求項２１のウイルスベクターにおいて、
前記ｓｉＲＮＡを発現するための核酸配列は、誘導性若しくは制御性プロモーターを有するものである。
【請求項23】
請求項２１のウイルスベクターにおいて、
前記ｓｉＲＮＡを発現するための核酸配列は、ヒトＵ６　ＲＮＡプロモーターの制御下でのｐｏｌｙＴ終止配列との操作可能な連結を持つ配列をコード化するセンスＲＮＡ鎖と、ヒトＵ６　ＲＮＡプロモーターの制御下でのｐｏｌｙＴ終止配列との操作可能な連結を持つ配列をコード化するアンチセンスＲＮＡ鎖とを有するものである。

【請求項24】
請求項２１の組換えウイルスベクターにおいて、
前記組換えウイルスベクターは、アデノウイルスベクター、アデノ随伴ウイルスベクター、レンチウイルスベクター、レトロウイルスベクター、及びヘルペスウイルスベクターからなる群から選択されるものである。
【請求項25】
請求項２１の組換えウイルスベクターにおいて、
前記組換えウイルスベクターは、水疱性口内炎ウイルス、狂犬病ウイルス、エボラウイルス、若しくはＭｏｋｏｌａウイルスからの表面タンパク質でシュードタイピングされているものである。
【請求項26】
請求項２４の組換えウイルスベクターにおいて、
前記組換えウイルスベクターは、アデノ随伴ウイルスベクターを有するものである。
【請求項27】
ｓｉＲＮＡと薬学的に許容可能な担体とを有する薬学的組成物であって、
前記ｓｉＲＮＡは、センスＲＮＡ鎖及びアンチセンスＲＮＡ鎖を有し、前記センスＲＮＡ鎖及びアンチセンスＲＮＡ鎖は、二本鎖を形成しており、前記センスＲＮＡ鎖は、ヒトＩＣＡＭ－１　ｍＲＮＡにおける約１９～約２５の隣接したヌクレオチドの標的配列と実質的に同一なヌクレオチド配列、若しくはそれらの選択的スプライシング体、変異体、或いは同族体を有する．薬学的組成物。
【請求項28】
請求項２７の薬学的組成物であって、この薬学的組成物は、さらに、
リポフェクチン、リポフェククミン、セルフェクチン、ポリカチオン、若しくはリポソームを有するものである。
【請求項29】
請求項１７のプラスミド若しくはその生理学的許容可能な塩類、及び薬学的に許容可能な担体を有する、薬学的組成物。
【請求項30】
請求項２９の薬学的組成物であって、この薬学的組成物は、さらに、
リポフェクチン、リポフェクタミン、セルフェクチン、ポリカチオン、若しくはリポソームを有するものである。
【請求項31】
請求項２１のウイルスベクター、及び薬学的に許容可能な担体を有する、薬学的組成物。
【請求項32】
ヒトＩＣＡＭ－１　ｍＲＮＡ、若しくはそれらの選択的スプライシング体、変異体、或いは同族体の発現を阻害する方法であって、
センスＲＮＡ鎖及びアンチセンスＲＮＡ鎖を有するｓｉＲＮＡの有効量を対象に投与する工程を有し、
前記センスＲＮＡ鎖及びアンチセンスＲＮＡ鎖は、二本鎖を形成し、前記センスＲＮＡ鎖は、ヒトＩＣＡＭ－１ｍＲＮＡにおける約１９～約２５の隣接したヌクレオチドの標的配列と実質的に同一なヌクレオチド配列．若しくはそれらの選択的スプライシング体、変異体、或いは同族体を有し、それにより前記ヒトＩＣＡＭ－１　ｍＲＮＡ、若しくはそれらの選択的スプライシング体、変異体、或いは同族体が分解される．方法。
【請求項33】
請求項３２の方法において、
前記対象は，ヒトである。
【請求項34】
請求項３２の方法において、
ヒトＩＣＭＡ－１　ｍＲＮＡ、若しくはそれらの選択的スプライシング体、変異体、若

しくは同族体の発現は、前記対象の片目若しくは両目において阻害されるものである。
【請求項35】
請求項３２の方法において、
ヒトＩＣＭＡ－１　ｍＲＮＡ、若しくはそれらの選択的スプライシング体、変異体、若しくは同族体の発現は、前記対象の網膜色素上皮細胞において阻害されるものである。
【請求項36】
請求項３２の方法において、
前記ｓｉＲＮＡの有効量は、約１ｎＭ～約１００ｎＭである。
【請求項37】
請求項３２の方法において、
前記ｓｉＲＮＡは、運搬試薬との組み合わせで投与されるものである。
【請求項38】
請求項３７の方法において、
前記運搬試薬は、リポフェクチン、リポフェクタミン、セルフェクチン、ポリカチオン、及びリポソームからなる群から選択されるものである。
【請求項39】
請求項３８の方法において、
前記運搬試薬は、リポソームである。
【請求項40】
請求項３９の方法において、
前記リポソームは、ＩＣＡＭ－１を発現している細胞を前記リポソームが標的とするためのリガンドを有するものである。
【請求項41】
請求項４０の方法において、
前記リガンドは、内皮細胞、上皮細胞、線維芽細胞、造血細胞、若しくは腫瘍細胞上の受容体に結合するものである。
【請求項42】
請求項４１の方法において、
前記内皮細胞は、網膜血管上皮細胞である。
【請求項43】
請求項４１の方法において、
前記造血細胞は、組織マクロファージ、分裂促進因子で刺激されたＴリンパ球芽細胞、扁桃腺における胚中心樹状細胞、リンパ節における胚中心樹状細胞、及びパイエル板における胚中心樹状細胞からなる群から選択されるものである。
【請求項44】
請求項４１の方法において、前記リガンドは、モノクローナル抗体を有するものである。
【請求項45】
請求項３９の方法において、
前記リポソームは、オプソニン化阻害部位で修飾されるものである。
【請求項46】
請求項４５の方法において、
前記オプソニン化阻害部位は、ＰＥＧ、ＰＰＧ、若しくはそれらの誘導体を有するものである。
【請求項47】
請求項３２の方法において、
前記ｓｉＲＮＡは、組換えプラスミドから発現されるものである。
【請求項48】
請求項３２の方法において、
前記ｓｉＲＮＡは、組換えウイルスベクターから発現されるものである。

【請求項49】
請求項４８の方法において、
前記組換えウイルスベクターは、アデノウイルスベクター、アデノ随伴ウイルスベクター、レンチウイルスベクター、若しくはヘルペスウイルスベクターを有するものである。
【請求項50】
請求項４９の方法において、
前記組換えウイルスベクターは、水疱性口内炎ウイルス、狂犬病ウイルス、エボラウイルス、若しくはＭｏｋｏｌａウイルスからの表面タンパク質でシュードタイピングされているレンチウイルスベクターである。
【請求項51】
請求項３２の方法において、
前記ｓｉＲＮＡは、経腸的投与経路によって投与されるものである。
【請求項52】
請求項５１の方法において、
前記経腸的投与経路は、経口、直腸、及び鼻腔内からなる群から選択されるものである。
【請求項53】
請求項３２の方法において、
前記ｓｉＲＮＡは、非経口的投与経路によって投与されるものである。
【請求項54】
請求項５３の方法において、
前記非経口的投与経路は、血管内投与、組織周辺及び組織内投与、皮下注射或いは沈着（ｄｅｐｏｓｉｔｉｏｎ）、皮下注入、眼内投与、及び、新血管新生部位での或いはその近くでの直接適用からなる群から選択されるものである。
【請求項55】
請求項５４の方法において、
前記血管内投与は、静脈内大量瞬時注射、静脈注入、動脈内大量瞬時注射、動脈内注入、及び脈管構造へのカテーテル滴下からなる群から選択されるものである。
【請求項56】
請求項５４の方法において、
前記組織周辺及び組織内注射は、腫瘍周辺注射、腫瘍内注射、網膜内注射、及び網膜下注射からなる群から選択されるものである。
【請求項57】
請求項５４の方法において、
前記眼内投与は、硝子体内、網膜内、網膜下、テノン嚢下（ｓｕｂｔｅｎｏｎ）、眼窩周辺及び眼窩後、トランス角膜及びトランス強膜投与を有するものである。
【請求項58】
請求項５４の方法において、
前記新血管新生部位での或いはその近くでの直接投与は、カテーテル、角膜ペレット、点眼剤、坐薬、多孔性物質を有する移植片、非多孔性物質を有する移植片、若しくはゼラチン物質を有する移植片による適用を有するものである。
【請求項59】
請求項５８の方法において、
前記新血管新生部位は眼にあり、新血管新生部位での或いはその近くでの前記直接投与は、点眼剤による適用を有するものである。
【請求項60】
対象における細胞接着若しくは細胞接着仲介性病態を阻害する方法であって、
センスＲＮＡ鎖及びアンチセンスＲＮＡ鎖を有するｓｉＲＮＡの有効量を対象に投与する工程を有し、前記センスＲＮＡ鎖は、ヒトＩＣＡＭ－１　ｍＲＮＡにおける約１９～約２５の隣接したヌクレオチドの標的配列と実質的に同一なヌクレオチド配列、若しくはそ

れらの選択的スプライシング体、変異体、或いは同族体を有する、方法。
【請求項61】
請求項６０の方法において、
前記細胞接着若しくは細胞接着仲介性病態は、ＡＩＤＳ関連性痴呆、アレルギー性結膜炎、アレルギー性鼻炎、アルツハイマー病、血管新生、抗原提示、喘息、アテローム性動脈硬化症、毒性腎炎、免疫性腎炎、接触性経皮過敏症、角膜／辺縁系損傷、糖尿病Ｉ型、糖尿病Ｉ型から生じる合併症、バゼドウ病、炎症性腸疾患、炎症性肺疾患、ウイルス感染の炎症性続発症、炎症性皮膚障害、同種移植片の拒絶、Ｔ細胞死滅、混合性リンパ球反応、及びＴ細胞仲介性Ｂ細胞分化などの免疫細胞相互作用、髄膜炎、多発性硬化症、多発性骨髄腫、心筋症、肺線維症、再灌流損傷、再狭窄、網膜炎、リュウマチ性関節炎、敗血性関節炎、脳卒中、腫瘍転移、及びｕｖｅｉｔｉｔｉｓからなる群から選択されるものである。
【請求項62】
請求項６１の方法において、
前記炎症性皮膚障害は、アレルギー性接触皮膚炎、固定薬剤皮疹、扁平苔癬、及び乾癬である。
【請求項63】
請求項６１の方法において、
前記同種移植片は、網膜、肝臓、若しくは骨髄移植である。
【請求項64】
請求項６２の方法において、
前記血管新生は、非病原性であり、脂肪組織の産生、コレステロールの産生、若しくは子宮内膜新血管新生に関連するものである。
【請求項65】
対象における血管新生病を治療する方法であって、
センスＲＮＡ鎖及びアンチセンスＲＮＡを有するｓｉＲＮＡの有効量をそのような治療を必要とする対象に投与する工程を有し、
前記センスＲＮＡ鎖及びアンチセンスＲＮＡ鎖は、二本鎖を形成し、前記センスＲＮＡ鎖は、ヒトＩＣＡＭ－１　ｍＲＮＡにおける約１９～約２５の隣接したヌクレオチドの標的配列と実質的に同一なヌクレオチド配列、若しくはそれらの選択的スプライシング体、変異体、或いは同族体を有し、それにより血管新生病に関連する血管新生が阻害される、方法。
【請求項66】
請求項６５の方法において、
前記血管新生病は、癌である。
【請求項67】
請求項６６の方法において、
前記癌は、乳癌、肺癌、頭部と頸部の癌、脳腫瘍、腹腔内腫瘍、大腸癌、結腸直腸癌、食道癌、胃腸癌、グリオーマ、肝癌、舌癌、神経芽細胞腫、骨肉腫、卵巣癌、膵臓癌、前立腺癌、網膜芽細胞腫、ウィルムス腫瘍、多発性骨髄腫、皮膚癌、リンパ腫、及び血液癌からなる群から選択されるものである。
【請求項68】
請求項６５の方法において、
前記血管新生病は、糖尿病性網膜症、及び年齢関連性黄斑変性症からなる群から選択されるものである。
【請求項69】
請求項６８の方法において、
前記血管新生病は、年齢関連性黄斑変性症である。
【請求項70】
請求項６５の方法において、

前記ｓｉＲＮＡは、血管新生病を治療するための薬剤との組み合わせで投与されるものであり、この薬剤は前記ｓｉＲＮＡとは異なるものである。
【請求項71】
請求項７０の方法において、
前記血管新生病は癌であり、前記薬剤は化学療法薬剤を有するものである。
【請求項72】
請求項７０の方法において、
前記化学療法薬剤は、シスプラチン、カルボプラチン、シクロホスファミド、５－フルオロウラシル、アドリアマイシン、ダウノルビシン、及びタモキシフェンからなる群から選択されるものである。
【請求項73】
請求項６５の方法において、
前記ｓｉＲＮＡは、血管新生病を治療するように設計された別の治療方法との組み合わせで、対象へ投与されるものである。
【請求項74】
請求項７３の方法において、
前記血管新生病は癌であり、前記ｓｉＲＮＡは放射療法、化学療法、若しくは手術との組み合わせで投与されるものである。
【請求項75】
対象における糖尿病Ｉ型から生じる合併症を治療するための方法であって、
センスＲＮＡ鎖及びアンチセンスＲＮＡ鎖を有するｓｉＲＮＡの有効量をそのような治療を必要としている対象に投与する工程を有し、
前記センスＲＮＡ鎖及びアンチセンスＲＮＡ鎖は、二本鎖を形成し、前記センスＲＮＡ鎖は、ヒトＩＣＡＭ－１　ｍＲＮＡにおける約１９～約２５の隣接したヌクレオチドの標的配列と実質的に同一なヌクレオチド配列、若しくはそれらの選択的スプライシング体、変異体、或いは同族体を有する、方法。
【請求項76】
請求項７５の方法において、
前記糖尿病Ｉ型から生じる合併症は、糖尿病性網膜症、糖尿病性神経障害、糖尿病性腎障害、及び大血管性疾患からなる群から選択されるものである。
【請求項77】
請求項７６の方法において、
前記大血管性疾患は、冠動脈疾患、脳血管疾患、若しくは末梢血管疾患である。
【発明の詳細な説明】
【技術分野】
【0001】
関連出願の相互参照
本明細書は、２００３年１月１６日出願の米国仮出願番号第６０／４４０，５７９号明細書に対して優先権を主張するものである。
【0002】
本発明は、特に、細胞間接着に関する疾患若しくは状態を治療するための、低分子干渉ＲＮＡによるＩＣＡＭ－１遺伝子発現の制御に関するものある。
【背景技術】
【0003】
多くの生理学的過程において、細胞は、他の細胞若しくは細胞外マトリックスに密接に接触し、接着していることが必要とされている。細胞－細胞、及び細胞－マトリックス間の相互作用は、細胞間接着分子若しくは"ＩＣＡＭｓ"のいくつかのファミリーを通じて仲介される。
【0004】
ＩＣＡＭ－１は、免疫グロブリンスーパーファミリーの１１０キログルトンメンバーであり（Ｓｉｍｍｏｎｓ　ｅｔ　ａｌ．，１９８８，Ｎａｔｕｒｅ（Ｌｏｎｄｏｎ）　３３

1:624～627）、限られた数の細胞上に発現し、刺激がなければ低レベルで発現する（Dustin et al.,1986 J.Immunol.137,245～254）。炎症介在物質で刺激した場合、異なる組織で様々な細胞タイプがそれらの表面に高レベルでICAM－1を発現する（Springer et al.,supra;Dustin et al.,supra;Rothlein et al.,1988,J.Immunol.141:1665～1669）。刺激されICAM－1を発現した細胞は、血管内皮細胞、胸腺や他の上皮細胞、及び線維芽細胞などの非造血性細胞、及び組織マクロファージ、分裂促進因子で刺激されたTリンパ球芽細胞、及び扁桃腺、リンパ節、パイエル板における胚中心樹状細胞などの造血性細胞を含む。ICAM－1誘導は、ICAM－1 mRNAの転写の増加を介して生じ（Simmons et al.,supra）、誘導後4時間で検出可能になり、誘導後16～24時間でピークとなる。

【0005】

In vitro研究によって、ICAM－1に対する抗体がサイトカイン活性化内皮細胞への白血球の接着を阻害することが示された（Boyd et al.,1988,Proc.Natl.Acad.Sci.USA 85:3095～3099;Dustin and Springer,1988,J.Cell.Biol.107:321～331）。従って、ICAM－1発現は、免疫細胞の炎症部位への血管外遊走に必要とされるように見える。ICAM－1に対する抗体は、T細胞死滅、混合性リンパ球反応、及びT細胞仲介性B細胞分化も阻害し、これはICAM－1がこれら同族細胞相互作用に必要であることを意味している（Boyd et al.,supra）。抗原が存在する場合のICAM－1の関与は、ICAM－1欠損マウスB細胞変異が抗原依存性T細胞増殖を刺激することができないことによって示された（Dang et al.,1990,J.Immunol.144:4082～4091）。反対に、マウスL細胞は、ヒトT細胞に対する抗原が存在するために、HLA－DRに加えてヒトICAM－1での形質移入を必要とする（Altmann et al.,1989,Nature(London)338:512～514）。従って、ICAM－1機能を阻害することによって、移植拒絶の間の免疫細胞の認識と活性を阻害することができ、リュウマチ性関節炎、喘息、及び再灌流損傷の動物モデルの治療に有効である。

【0006】

ICAM－1の発現は、アレルギー性接触皮膚炎、固定薬剤皮疹、扁平苔癬、及び乾癬などの炎症性皮膚障害にも関連している（Ho et al.,1990,J.Am.Acad.Dermatol.,22:64～68;Griffiths and Nickoloff,1989,Am.J.Pathology 135:1045～1053;Lisby et al.,1989,Br.J.Dermatol.120:479～484;Shiohara et al.,1989,Arch.Dermatol.125:1371～1376）。さらに、ICAM－1発現は、リウマチ性関節炎の患者の滑膜において（Hale et al.,1989,Arth.Rheum.,32:22～30）、糖尿病の膵臓B細胞において（Campbell et al.,1989,P.N.A.S.USA 86:4282～4286）、バゼドウ病の患者の甲状腺濾胞細胞において（Weetman et al.,1989,J.Endocrinol.122:185～191）、腎臓及び肝臓同種移植片拒絶において（Faull and Russ,1989,Transplantation 48:226～230:Adams et al.,1989,Lancet 1122～1125）、及び炎症性腸疾患（IBD）組織において（Springer T.,1990,Nature 346:425～34）も検出された。

【0007】

ICAM－1発現は、既存の血管からの新規血管の形成である血管新生においても関係がある。血管新生とは、細胞遊走、増殖、及び分化に関連した内皮細胞遺伝子発現の特性の変化を伴う複雑な過程であり、親血管の基底膜の局在性分解から始まるものである。次にその内皮細胞は親血管から間質性細胞外マトリックス（ECM）へと遊走し、ECMの

内皮細胞の継続した遊走や増殖に起因して伸張する毛細血管出芽を形成する。血管新生の間の内皮細胞とＥＣＭとの相互作用は、部分的にはＩＣＡＭ－１発現の増加によって引き起こされる細胞－マトリックス接触の変化を必要とする。

【0008】

異常血管新生、若しくは新規血管の病的な増殖は、様々な状態を意味している。これらの状態としては、糖尿病性網膜症、乾癬、滲出性若しくは"濡れた"年齢関連性黄斑変性症（"ＡＭＤ"）、リュウマチ性関節炎や他の炎症疾患、及びほとんどの癌が挙げられる。ＡＭＤは特に、臨床的に重要な血管新生病である。この状態は、高齢者の片目若しくは両目における脈絡叢新生血管によって特徴付けられ、先進工業国における失明の主要な原因である。

【0009】

糖尿病Ｉ型で共通に見られるいくつかの合併症にも、ＩＣＡＭ－１の発現が関与している。例えば毛細血管内皮への白血球のＩＣＡＭ－１仲介性接着（"ｌｅｕｋｏｓｔａｓｉｓ"とも呼ばれる）は、糖尿病の特定の組織、例えば網膜、末梢神経、及び腎臓などにおいて微小血管虚血を引き起こすことができる。これはこれらの組織の毛細非灌流を生じ、次に糖尿病性網膜症（Ｍｉｙａｍｏｔｏ Ｋ ｅｔ ａｌ．，（２０００），Ａｍ．Ｊ．Ｐａｔｈｏｌ．１５６:１７３３～１７３９；Ｍｉｙａｍｏｔｏ Ｋ ｅｔ ａｌ．（１９９９），Ｐ．Ｎ．Ａ．Ｓ ＵＳＡ ９６:１０８３６～１０８４）、神経障害（Ｊｕｄｅ ＥＢ ｅｔ ａｌ．（１９９８），Ｄｉａｂｅｔｏｌｏｇｉａ ４１：３３０～６）、若しくは腎障害を導く。Ｍｉｙａｍｏｔｏら（１９９９，Ｐ．Ｎ．Ａ．Ｓ ＵＳＡ ９６：１０８３６～１０８４１）は、ＩＣＡＭ－１仲介性ｌｅｕｋｏｓｔａｓｉｓの阻害によって糖尿病に関連した網膜異常を阻害することができると示唆した。しかしながら、少なくとも１つの研究では、糖尿病の"ウィスター系脂肪"ラットモデルにおける糖尿病性腎障害の発展には、糸球体におけるＩＣＡＭ－１発現の関与は見られないと報告した（Ｍａｔｓｕｉ Ｈ ｅｔ ａｌ．（１９９６），Ｄｉａｂｅｔｅｓ Ｒｅｓ．Ｃｌｉｎ．Ｐｒａｃｔ．３２：１～９）。

【0010】

ＩＣＡＭ－１は、糖尿病Ｉ型における大血管性疾患（例えば冠動脈疾患、脳血管疾患、及び末梢血管疾患など）の発病にも関与しており、これは部分的には、アテローム性動脈硬化症の促進、及び血栓症の増加に起因するものである。例えば、ＩＣＡＭ－１は、アテローム性硬化硬化型プラークにおいて発見され、糖尿病におけるアテローム性動脈硬化症の開始と発展を伴っているようである（Ｊｕｄｅ ＥＢ ｅｔ ａｌ．（２００２），Ｅｕｒ．Ｊ．Ｉｎｔｅｒｎ．Ｍｅｄ．１３：１８５～１８９）。

【0011】

従って、ＩＣＡＭ－１は、正常過程及び病態生理学的過程の両方において絶対不可欠な役割を担っている（Ｓｐｒｉｎｇｅｒ ｅｔ ａｌ．，１９８７，Ａｎｎ．Ｒｅｖ．Ｉｍｍｕｎｏｌ．５：２２３～２５２）。従って、ＩＣＡＭ－１機能或いは発現を阻害することによる細胞接着を媒介するための戦略が開発されてきた。そのような戦略では一般的に、抗ＩＣＡＭ－１抗体、ＩＣＡＭ－１結合を競合的に阻害するリガンド、若しくはＩＣＡＭ－１　ｍＲＮＡに対するアンチセンス核酸分子を用いる。しかしながら、そのような治療で用いられた作用因子は、ＩＣＡＭ－１の化学量論的な減少のみ産生し、一般的には罹患細胞若しくは活性化細胞によるＩＣＡＭ－１の異常な高産生によって圧倒される。従って、これらの戦略によって達成された結果は満足できるものではなかった。

【0012】

ＲＮＡ干渉（以後"ＲＮＡｉ"とする）は、転写後遺伝子制御の方法であり、多くの真核生物を通じて保存されているものである。ＲＮＡｉは、すべての細胞に存在する、短い（例えば、<３０ヌクレオチド）二本鎖ＲＮＡ（"ｄｓＲＮＡ"）分子によって誘導される（Ｆｉｒｅ Ａ ｅｔ ａｌ．（１９９８），Ｎａｔｕｒｅ ３９１：８０５～８１１）。"低分子干渉ＲＮＡ"若しくは"ｓｉＲＮＡ"と呼ばれるこれらの短いｄｓＲＮＡ分子は、前記ｓｉＲＮＡと配列相同性を有しているメッセンジャーＲＮＡｓ（"ｍＲＮＡｓ"）を破壊す

ることで1ヌクレオチドの範囲での分解を引き起こす（Ｅｌｂａｓｈｉｒ ＳＭ ｅｔ ａｌ．（２００１），Ｇｅｎｅｓ Ｄｅｖ．１５：１８８～２００）．このｓｉＲＮＡと標的ｍＲＮＡは、前記標的ｍＲＮＡを切断する"ＲＮＡ誘導サイレンシング複合体"若しくは"ＲＩＳＣ"に結合すると考えられている。ｓｉＲＮＡは明らかに複数一回転（ｍｕｌｔｉｐｌｅ－ｔｕｒｎｏｖｅｒ）酵素と同様に再利用され、１つのｓｉＲＮＡ分子は約１００ｍＲＮＡ分子の切断を誘導することが可能である。従って、ｍＲＮＡのｓｉＲＮＡ仲介ＲＮＡｉ分解は、標的遺伝子の発現を阻害するための現在利用可能な技術に比べてより効果的である。

【0013】

Ｅｌｂａｓｈｉｒ ＳＭら（（２００１），ｓｕｐｒａ）は、２１及び２２ヌクレオチド長で短い３′オーバーハングを有する合成ｓｉＲＮＡは、ショウジョウバエ細胞可溶化液中の標的ｍＲＮＡのＲＮＡｉを誘導することができることを示した。培養哺乳類細胞でも合成ｓｉＲＮＡによるＲＮＡｉが見られ（Ｅｌｂａｓｈｉｒ ＳＭ ｅｔ ａｌ．（２００１）Ｎａｔｕｒｅ ４１１：４９４～４９８）、さらに、合成ｓｉＲＮＡによって誘導されたＲＮＡｉ分解は最近、生きているマウスでも示された（ＭｃＣａｆｆｒｅｙ ＡＰ ｅｔ ａｌ．（２００２），Ｎａｔｕｒｅ，４１８：３８～３９；Ｘｉａ Ｈ ｅｔ ａｌ．（２００２），Ｎａｔ．Ｂｉｏｔｅｃｈ．２０：１００６～１０１０）．ｓｉＲＮＡ誘導性ＲＮＡｉ分解の治療上の潜在能力は、ＨＩＶ－１感染のｓｉＲＮＡ指向性（ｓｉＲＮＡ－ｄｉｒｅｃｔｅｄ）阻害（Ｎｏｖｉｎａ ＣＤ ｅｔ ａｌ．（２００２），Ｎａｔ．Ｍｅｄ．８：６８１～６８６）、及び神経毒性ポリグルタミン疾患タンパク質発現の減少（Ｘｉａ Ｈ ｅｔ ａｌ．（２００２），ｓｕｐｒａ）を含む、最近のいくつかのｉｎ ｖｉｔｒｏ研究で実証された。

【0014】

従って、ＩＣＡＭ－１仲介性細胞接着を効果的に減少する若しくは阻害するＩＣＡＭ－１の発現を阻害する、触媒的若しくは半化学量論的量の作用因子が必要とされている。

【発明の開示】

【発明の効果】

【0015】

本発明は、特にＩＣＡＭ－１遺伝子からのｍＲＮＡのＲＮＡｉ誘導分解を標的にし、それを引き起こすｓｉＲＮＡに関するものである。本発明のｓｉＲＮＡ化合物や組成物は、細胞接着及び細胞接着仲介性病態を治療するために用いられる。特に、本発明のｓｉＲＮＡは、例えば、癌性腫瘍、年齢関連性黄斑変性症、及び他の血管新生病の治療などにおいて、血管新生を阻害するために有用である。

【0016】

従って、本発明は、ヒトＩＣＡＭ－１ｍＲＮＡ、若しくはその選択的スプライシング型、変異体若しくは同族体を標的とする単離されたｓｉＲＮＡを提供する。前記ｓｉＲＮＡは、ＲＮＡ二本鎖を形成するセンスＲＮＡ鎖とアンチセンスＲＮＡ鎖とを有する。前記センスＲＮＡ鎖は、標的ｍＲＮＡにおける近接した約１９～約２５ヌクレオチドの標的配列と実質的に同一なヌクレオチド配列を有している。

【0017】

本発明は、本発明のｓｉＲＮＡを発現する組換えプラスミドとウイルス性ベクター、さらに本発明のｓｉＲＮＡと薬学的に許容可能な担体とを有する薬学的組成物も提供する。

【0018】

本発明はさらに、ヒトＩＣＡＭ－１　ｍＲＮＡ、若しくはその選択的スプライシング型、変異体、若しくは同族体の発現を阻害する方法を提供し、これは標的ｍＲＮＡが分解されるように本発明のｓｉＲＮＡの有効量を対象に投与する工程を有するものである。

【0019】

本発明はさらに、細胞接着若しくは細胞接着仲介性病態を治療する方法も提供し、これはヒトＩＣＡＭ－１　ｍＲＮＡ、若しくはその選択的スプライシング型、変異体、若しくは同族体を標的としたｓｉＲＮＡの有効量をそのような治療を必要としている対象に投与

する工程を有するものである。
【0020】
本発明はさらに、対象における血管新生を阻害する方法も提供し、これはヒトＩＣＡＭ－１　ｍＲＮＡ、若しくはその選択的スプライシング型、変異体若しくは同族体を標的としたｓｉＲＮＡの有効量を対象に投与する工程を有するものである。
【0021】
本発明はさらに、対象における糖尿病Ｉ型による合併症を治療する方法も提供し、これはヒトＩＣＡＭ－１　ｍＲＮＡ、若しくはその選択的スプライシング型、変異体若しくは同族体を標的としたｓｉＲＮＡの有効量をそのような治療を必要とする対象に投与する工程を有するものである。
【発明を実施するための最良の形態】
【0022】
特に指示しない限り、この中の全ての核酸配列は５’から３’方向に示されているものとする。また、核酸配列における全てのデオキシリボヌクレオチドは、大文字で表されており（例えばデオキシチミジンは"Ｔ"である）、核酸配列におけるリボヌクレオチドは、小文字で表されている（例えばウリジンは"ｕ"である）。
【0023】
ＩＣＡＭ－１　ｍＲＮＡを標的にしたｓｉＲＮＡを有する組成物及び方法は、細胞接着及び細胞接着仲介性病態の阻害若しくは予防において有利に用いられる。ここで用いられたように"細胞接着及び細胞接着仲介性病態"とは、疾患若しくは状態を発病する及び／若しくは維持するために、ある細胞と他の細胞との若しくはある細胞と細胞外マトリックスとのＩＣＡＭ－１仲介性接着を必要とするあらゆる疾患若しくは状態を言及する。例えば、血管新生は内皮細胞の細胞外マトリックスとのＩＣＡＭ－１仲介性接着を必要とするように、当業者にとってそのような疾患や状態は周知である。さらに、炎症部位への免疫細胞の血管外遊走は、白血球のサイトカイン活性化内皮細胞へのＩＣＡＭ－１仲介性接着を必要とする。他の細胞接着及び細胞接着仲介性病態としては、ＡＩＤＳ関連性痴呆、アレルギー性結膜炎、アレルギー性鼻炎、アルツハイマー病、血管新生（病原性若しくは非病原性血管新生を含む）、抗原提示、喘息、アテローム性動脈硬化症、ある種の毒性及び免疫性腎炎、接触性経皮過敏症、角膜／辺縁系損傷、糖尿病Ｉ型、例えば糖尿病性網膜症、糖尿病性神経障害、糖尿病性腎障害、及び大血管性疾患などの糖尿病Ｉ型から生じる合併症、バゼドウ病、炎症性腸疾患（潰瘍性大腸炎やクローン病を含む）、炎症性腸疾患、ウイルス感染の炎症性続発症、炎症性皮膚障害（例えば、アレルギー性接触皮膚炎、固定薬剤皮疹、扁平苔癬、及び乾癬など）、腎臓、肝臓及び骨髄移植を含む移植（同種移植）拒絶における免疫細胞認識と活性、免疫細胞相互作用（例えば、Ｔ細胞死滅、混合性リンパ球反応、及びＴ細胞仲介性Ｂ細胞分化など）、髄膜炎、多発性硬化症、多発性骨髄腫、心筋症、肺線維症、再灌流損傷、再狭窄、網膜炎、リュウマチ性関節炎、敗血性関節炎、脳卒中、腫瘍増殖と転移、及びｕｖｅｉｔｉｔｉｓを含む。
【0024】
本発明のｓｉＲＮＡは、ＩＣＡＭ－１遺伝子のタンパク質産物が産生しない、若しくは少量で産生されるように、ＩＣＡＭ－１　ｍＲＮＡのＲＮＡｉ仲介性分解を引き起こす。ＩＣＡＭ－１遺伝子産物は、特定の細胞間若しくは細胞－ＥＣＭ接着現象を必要とするため、ＩＣＡＭ－１　ｍＲＮＡのｓｉＲＮＡ仲介性分解は細胞間若しくは細胞－ＥＣＭ接着を阻害する。従って、細胞接着若しくは細胞接着仲介性病態は、本発明のｓｉＲＮＡを用いてＩＣＡＭ－１　ｍＲＮＡのＲＮＡｉ分解を誘導することによって治療され得る。
【0025】
従って、本発明は単離されたｓｉＲＮＡを提供し、これは、前記標的ｍＲＮＡを標的とした約１７ヌクレオチド～２９ヌクレオチド長の、好ましくは約１９～２５ヌクレオチド長の短い二本鎖ＲＮＡを有するものである。前記ｓｉＲＮＡは、標準ワトソン－クリック塩基対相互作用（ここでは以後"塩基対の（ｂａｓｅ－ｐａｉｒｅｄ）"とする）によってアニールされたセンスＲＮＡ鎖と相補的なアンチセンスＲＮＡ鎖とを有している。以下に

より詳細に記載されているように、前記センス鎖は、前記標的ｍＲＮＡ内に含まれる標的配列と実質的に同一な核酸配列を有している。

【0026】

ここで用いられたように、前記標的ｍＲＮＡ内に含まれる標的配列と"実質的に同一な"核酸配列とは、標的配列と同一な、若しくは１若しくはそれ以上のヌクレオチドが前記標的配列とは異なっている、核酸配列である。標的配列に実質的に同一な核酸配列を有する本発明のセンス鎖は、そのようなセンス鎖を有するｓｉＲＮＡが前記標的配列に含まれるｍＲＮＡのＲＮＡｉ仲介性分解を誘導するという点で特徴付けられる。例えば、本発明のｓｉＲＮＡは、標的ｍＲＮＡのＲＮＡｉ仲介性分解がｓｉＲＮＡによって誘導される限りで、１、２、或いは３、若しくはそれ以上のヌクレオチドが標的配列と異なっている核酸配列を有しているセンス鎖を有することができる。

【0027】

本発明のｓｉＲＮＡのセンス及びアンチセンス鎖は、２つの相補的で一本鎖ＲＮＡ分子を有することができる、若しくは２つの相補的な部分は塩基対であり、一本鎖"ヘアピン"領域で共有結合的にリンクしている、１分子を有することができる。あらゆる理論に束縛されることなく、後者タイプのｓｉＲＮＡ分子のヘアピン領域は、"ダイサー"タンパク質（若しくはその等価物）によって細胞質を切断され、２つの独立した塩基対ＲＮＡ分子を形成すると信じられている（Ｔｕｓｃｈｌ，Ｔ．（２００２），ｓｕｐｒａを参照のこと）。

【0028】

ここで用いられたように"単離された"とは、合成の、若しくはヒトの介入を通じて天然状態から変化した或いは除去されたことを意味する。例えば、生きた動物中に天然に存在するｓｉＲＮＡは"単離され"てないが、合成ｓｉＲＮＡ、若しくはその天然状態の共存物質から部分的に或いは完全に分離されたｓｉＲＮＡは"単離され"ている。単離されたｓｉＲＮＡは、実質的に精製された形態で存在し得る、若しくは例えば、ｓｉＲＮＡが運搬された細胞などの非天然環境中に存在し得る。一例として、天然過程により細胞内部に生産されるが、"単離された"前駆分子から生成されるｓｉＲＮＡは、"単離された"分子である。従って、単離されたｄｓＲＮＡ若しくはタンパク質は、標的細胞へ導入され得、ダイサータンパク質（若しくはその等価物）によって単離されたｓｉＲＮＡ内へプロセシングされる。

【0029】

ここで用いられたように"標的ｍＲＮＡ"とは、ヒトＩＣＡＭ－１　ｍＲＮＡ、ヒトＩＣＡＭ－１　ｍＲＮＡの変異体或いは選択的スプライシング体、若しくは同族ＩＣＡＭ－１遺伝子からのｍＲＮＡを意味する。ヒトＩＣＡＭ－１　ｍＲＮＡ配列は，ｃＤＮＡに相当するものとして、配列ＩＤ番号：１に示されている。当業者は、ｃＤＮＡ配列はｍＲＮＡ配列と同等であり、ここにおいて同じ目的、すなわち、ＩＣＡＭ－１の発現を阻害するためのｓｉＲＮＡの産生で用いることが可能であることを理解するであろう。

【0030】

ここで用いられたように、ヒトＩＣＡＭ－１と"同族な"遺伝子若しくはｍＲＮＡは、ヒトＩＣＡＭ－１と相同な別の哺乳類種からの遺伝子若しくはｍＲＮＡである。例えば，マウスからの同族ＩＣＡＭ－１　ｍＲＮＡは，ｃＤＮＡに相当するものとして，配列ＩＤ番号：２に示されている。

【0031】

ヒトＩＣＡＭ－１遺伝子から転写されたｍＲＮＡは、選択的スプライシング体に対して本分野ではよく知られた技術を用いて解析され得る。そのような技術は，逆転写ポリメラーゼ連鎖反応（ＲＴ－ＰＣＲ）、ノーザンブロッティング、及びｉｎ－ｓｉｔｕハイブリダイゼーションなどが含まれる。ｍＲＮＡ配列を解析するための技術は、例えば、Ｂｕｓｔｉｎｇ ＳＡ（２０００），Ｊ．Ｍｏｌ．Ｅｎｄｏｃｒｉｎｏｌ．２５：１６９～１９３に記載されており、そこで開示された全てはこの参照によって本明細書に組み込まれる。選択的スプライシングｍＲＮＡを同定するための代表的な技術は、以下にも記載されて

いる。

【0032】

例えば、所定の疾患遺伝子に関連したヌクレオチド配列を含むデータベースは、選択的スプライシングｍＲＮＡを同定するために使用することができる。そのようなデータベースは、ＧｅｎＢａｎｋ、Ｅｍｂａｓｅ、ｔｈｅ Ｃａｎｃｅｒ Ｇｅｎｏｍｅ Ａｎａｔｏｍｙ Ｐｒｏｊｅｃｔ（ＣＧＡＰ）データベースが含まれる。例えば、このＣＧＡＰデータベースは、様々なタイプのヒト癌からの発現遺伝子配列（ＥＳＴｓ）を含む。ＩＣＡＭ－１遺伝子からのｍＲＮＡ若しくは遺伝子配列は、選択的スプライシングｍＲＮＡｓを表すＥＳＴｓが発見されたかどうかを決定するためにそのようなデータベースをクエリーするために使用することができる。

【0033】

"ＲＮＡｓｅ（ＲＮＡ分解酵素）保護"と呼ばれる技術は、選択的スプライシングＩＣＡＭ－１　ｍＲＮＡを同定するために使用することができる。ＲＮＡｓｅ保護は、例えば、ＩＣＡＭ－１を発現するように誘導された細胞などの他の細胞に由来したＲＮＡとハイブリダイズされた合成ＲＮＡへの遺伝子配列の翻訳を必要とするものである。次にこのハイブリダイズされたＲＮＡは、ＲＮＡ：ＲＮＡハイブリッドミスマッチを認識する酵素をともにインキュベートされる。予想より小さい断片（フラグメント）は、選択的スプライシングｍＲＮＡｓの存在を意味している。推定上の選択的スプライシングｍＲＮＡｓは、当業者にはよく知られた方法によって、クローニングされ、配列決定される。

【0034】

ＲＴ－ＰＣＲも、選択的スプライシングＩＣＡＭ－１　ｍＲＮＡｓを同定するために使用することができる。ＲＴ－ＰＣＲにおいて、活性化白血球からのｍＲＮＡ、炎症性腸疾患組織の細胞からのｍＲＮＡ、若しくはＩＣＡＭ－１を発現すると知られている他の組織の細胞からのｍＲＮＡは、当業者にはよく知られた方法を用いて、酵素逆転写によってｃＤＮＡへ変換される。次にこのｃＤＮＡの全コード配列は、３’非翻訳領域に位置付けられた前方（フォワード）プライマーと、５’非翻訳領域に位置付けられた後方（リバース）プライマーとを用いるＰＣＲによって増幅される。増幅産物は、例えば、正常なスプライシングｍＲＮＡから発現した産物のサイズと増幅された産物のサイズとを比較すること（すなわち、アガロースゲル電気泳動）によって、選択的スプライシング体のための解析ができる。前記増幅産物のサイズにおけるどんな変化も、選択的スプライシングを意味するものである。

【0035】

変異ＩＣＡＭ－１遺伝子から産生されたｍＲＮＡも、ＩＣＡＭ－１選択的スプライシング体を同定するための上述された技術を用いて、容易に解析され得る。ここで用いられたように"変異ＩＣＡＭ－１遺伝子若しくはｍＲＮＡ"は、ここで説明されたＩＣＡＭ－１配列からの配列とは異なるヒトＩＣＡＭ－１遺伝子、若しくはｍＲＮＡを含む。従って、ＩＣＡＭ－１遺伝子対立遺伝子型、及びそれらからのｍＲＮＡ産物は、本発明の目的のために"変異体"として考えられる。

【0036】

ヒトＩＣＡＭ－１　ｍＲＮＡは、そのそれぞれの選択的スプライシング体、同族体、若しくは変異体と共通した標的配列を含む可能性があると理解されている。従って、そのような共通の標的配列を有しているＩつのｓｉＲＮＡは、共通の標的配列を含むそれらの異なるｍＲＮＡｓのＲＮＡｉ仲介性分解を誘導することができる。

【0037】

本発明のｓｉＲＮＡは、１若しくはそれ以上のヌクレオチドの付加、欠損、置換及び／若しくは変更による天然由来のＲＮＡとは異なる変化ＲＮＡと同様に、部分的に精製されたＲＮＡ、実質的に純粋なＲＮＡ、合成ＲＮＡ、若しくは組換え的に生成されたＲＮＡを有し得る。そのような変化は、例えば、ｓｉＲＮＡの末端へ或いは１若しくはそれ以上の内部ヌクレオチドへの非ヌクレオチド物質の付加、ｓｉＲＮＡをヌクレアーゼ消化耐性にするような修飾（例えば、２’－置換リボヌクレオチドの使用、若しくは糖－リン酸バッ

クボーンの修飾など）、若しくは、ｓｉＲＮＡの１若しくはそれ以上のヌクレオチドのデオキシリボヌクレオチドでの置換を含むことができる。血清、涙液（ｌａｃｈｒｙｍａｌ　ｆｌｕｉｄ）、若しくは他のヌクレアーゼが豊富な環境にさらされたｓｉＲＮＡ、若しくは局所的に運搬された（例えば目薬などで）ｓｉＲＮＡは、ヌクレアーゼ分解に対する耐性が増加するように変えられていることが好ましい。例えば、血管内に、若しくは眼に局所的に投与されたｓｉＲＮＡは、１若しくはそれ以上のホスホロチオエート連鎖を有し得る。

【００３８】

一本鎖若しくは両鎖の本発明のｓｉＲＮＡは、３’オーバーハングも有することができる。ここで用いられたように、”３’オーバーハング”とは、ＲＮＡ鎖の３’末端から伸長した少なくとも１つの不対ヌクレオチドを意味する。

【００３９】

従って、１実施形態において、本発明のｓｉＲＮＡは、１～約６ヌクレオチド（リボヌクレオチド若しくはデオキシヌクレオチドを含む）長、好ましくは１～約５ヌクレオチド長、より好ましくは１～約４ヌクレオチド長、特に好ましくは約２～約４ヌクレオチド長、からなる少なくとも１つの３’オーバーハングを有している。

【００４０】

ｓｉＲＮＡ分子の二本鎖は３’オーバーハングを有しているという１実施形態において、前記オーバーハングの長さは、各鎖で同じ若しくは異なってもよい。最も好ましい実施形態において、前記３’オーバーハングは、ｓｉＲＮＡの両方の鎖に存在し、２ヌクレオチド長である。例えば、本発明のｓｉＲＮＡの各鎖は、ジチミジル酸（”ＴＴ”）若しくはジウリジル酸（”ｕｕ”）の３’オーバーハングを有することができる。

【００４１】

本発明のｓｉＲＮＡの安定性を増強するために、前記３’オーバーハングも分解に対して安定化され得る。１実施形態において、前記オーバーハングは、アデノシン若しくはグアノシンヌクレオチドなどのプリンヌクレオチドを含むことによって安定化される。あるいは、例えば３’オーバーハングのウリジンヌクレオチドの２‘－デオキシチミジンでの置換などの、修飾された類似体によるピリミジンヌクレオチドの置換は、耐容性を示し、ＲＮＡｉ分解の効率に影響を与えないものである。特に、２’－デオキシチミジンに２’ヒドロキシルが存在しないと、組織培養液中の３’オーバーハングのヌクレアーゼ耐性が著しく増強する。

【００４２】

いくつかの実施形態において、本発明のｓｉＲＮＡは、配列ＡＡ（Ｎ１９）ＴＴ若しくはＮＡ（Ｎ２１）を有しており、ここにおいてＮはあらゆるヌクレオチドである。これらのｓｉＲＮＡは、約３０～７０％　Ｇ／Ｃを有し、好ましくは約５０％　Ｇ／Ｃを有している。センスｓｉＲＮＡ鎖の配列は、それぞれ（Ｎ１９）ＴＴ若しくはＮ２１（すなわち、３～２３位）と一致するものである。後者の場合において、前記センスｓｉＲＮＡの３’末端はＴＴに変換される。この配列変換の理論的な解釈としては、センス及びアンチセンス鎖３’オーバーハングの配列組成に関して対照的な二本鎖を生じることである。次に前記アンチセンスＲＮＡ鎖は、前記センス鎖の１～２１位を補完するものとして合成される。

【００４３】

これらの実施例における２３－ｎｔセンス鎖の１位は、アンチセンス鎖による配列特異的な方法では認識されないので、前記アンチセンス鎖のほとんどの３’－ヌクレオチド残基は意図的に選択され得る。しかしながら、前記アンチセンス鎖の最後から２番目のヌクレオチド（いずれの実施形態においても２３－ｎｔセンス鎖の２位を補完するもの）は、一般的に標的配列を補完するものである。

【００４４】

別の実施形態において、本発明のｓｉＲＮＡは、配列ＮＡＲ（Ｎ１７）ＹＮＮを有しており、ここにおいてＲはプリン（例えばＡ若しくはＧ）であり、Ｙはピリミジン（例えば

Ｃ若しくはＵ／Ｔ）である。従って、本実施形態の２１－ｎｔセンス及びアンチセンスＲＮＡ鎖のそれぞれは、一般的にプリンヌレオチドで始まる。最初に転写されたヌクレオチドがプリンである場合、ｐｏｌ　ＩＩＩプロモーターからのＲＮＡｓの発現が唯一効果的であると信じられているように、そのようなｓｉＲＮＡは、標的部位を変更することなく、ｐｏｌ　ＩＩＩ発現ベクターから発現され得る。

【0045】

本発明のｓｉＲＮＡは、あらゆる標的ｍＲＮＡ配列（"標的配列"）における一続きの約１９～２５の近接したヌクレオチドを標的とし得る。ｓｉＲＮＡのための標的配列を選択するための技術は、例えば、Ｔｕｓｃｈｌ Ｔらの"Ｔｈｅ ｓｉＲＮＡ Ｕｓｅｒ Ｇｕｉｄｅ"（２００２年１０月１１日改訂）に示されており、そこに開示されている全てはこの参照によって本明細書に組み込まれる。"Ｔｈｅ ｓｉＲＮＡ Ｕｓｅｒ Ｇｕｉｄｅ"は、Ｔｈｏｍａｓ　Ｔｕｓｃｈｌ博士（Ｄｅｐａｒｔｍｅｎｔ ｏｆ Ｃｅｌｌｕｌａｒ Ｂｉｏｃｈｅｍｉｓｔｒｙ，ＡＧ１０５，Ｍａｘ－Ｐｌａｎｃｋ－Ｉｎｓｔｉｔｕｔｅ ｆｏｒ Ｂｉｏｐｈｙｓｉｃａｌ Ｃｈｅｍｉｓｔｒｙ，３７０７７ Ｇｏｔｔｉｎｇｅｎ，Ｇｅｒｍａｎｙ）によって維持されているウェブサイトのワールドワイドウェブ（ｗｗｗ）上で入手可能であり、Ｍａｘ　Ｐｌａｎｃｋ　Ｉｎｓｔｉｔｕｔｅのウェブサイトにアクセスし、キーワード"ｓｉＲＮＡ"で検索ことによって発見することができる。従って、本発明のｓｉＲＮＡのセンス鎖は、標的ｍＲＮＡにおけるあらゆる近接した一続きの約１９～約２５ヌクレオチドと実質的に同一なヌクレオチド配列を有している。

【0046】

一般的に、標的ｍＲＮＡの標的配列は、標的ｍＲＮＡに対応する特定の、好ましくは開始コドンから５０～１００ｎｔ下流（すなわち３’方向）から始まるｃＤＮＡ配列から選択され得る。しかしながら前記標的配列は、５’或いは３’非翻訳領域に、若しくは開始コドンに近接する領域に位置付けられ得る。例えば、ＩＣＡＭ－１ｃＤＮＡ配列中の適切な標的配列としては、

【0047】

【化1】

GTTGTTGGGCATAGAGACC　　（配列ＩＤ番号：３）

【0048】

従って、この配列を標的とし、各鎖に３’ｎｕオーバーハング（オーバーハングは太字で表している）を有する本発明のｓｉＲＮＡとしては、

【0049】

【化2】

5'- guuguugggcauagagaccuu - 3'　（配列ＩＤ番号：４）

3' - uucaacaacccguaucucugg - 5'　（配列ＩＤ番号：５）

である。

【0050】

配列ＩＤ番号：３を標的としているが、各鎖に３’ＴＴオーバーハング（オーバーハングは太字で表している）を有する本発明のｓｉＲＮＡとしては、

【0051】

【化3】

5'- guuguugggcauagagaccTT - 3'（配列ＩＤ番号：６）

3' -TTcaacaacccguaucucugg - 5'　（配列ＩＤ番号：７）

である。

【0052】

本発明のｓｉＲＮＡが由来され得る他のＩＣＡＭ－１標的配列は、表１に記載された配列と、配列ＩＤ番号：２０～９４に記載された配列とを含む。ここに記載された前記標的配列は、ヒトＩＣＡＭ－１ｃＤＮＡを参照とし、従ってそれらの配列は"T"で示されたデオキシチミジンを含むことが理解される。当業者は、ＩＣＡＭ－１ｍＲＮＡの実際の標的配列において、前記デオキシチミジンがウリジン（"u"）で置換されることを理解するであろう。同様に、本発明のｓｉＲＮＡ内に含まれる標的配列も、デオキシチミジンの代わりにウリジンを含むことが可能である。

【0053】

【表1】

Table 1－ＩＣＡＭ－１標的配列

標的配列	配列ＩＤ番号：
GGAGTTGCTCCTGCCTGGG	8
CCGGAAGGTGTATGAACTG	9
CTGAGCAATGTGCAAGAAG	10
TGTGCTATTCAAACTGCCC	11
CCTTCCTCACCGTGTACTG	12
CGGGTGGAACTGGCACCCC	13
CCTTACCCTACGCTGCCAG	14
CCTCACCGTGGTGCTGCTC	15
CGGGAGCCAGCTGTGGGGG	16
TTTCTCGTGCCGCACTGAA	17
CTGGACCTGCGGCCCCAAG	18
GGCCTCAGTCAGTGTGACC	19

【0054】

本発明のｓｉＲＮＡは、当業者に知られた多くの技術を用いて得ることができる。例え

ば、Ｔｕｓｃｈｌらによる米国公開公報第２００２／００８６３５６号に記載されたｔｈｅ Ｄｒｏｓｏｐｈｉｌａ ｉｎ ｖｉｔｒｏシステム（この全体の開示はこの参照により本明細書に組み込まれる。）などのｓｉＲＮＡは従来技術において知られた方法を用いて、化学的に合成され得る、若しくは組換え的に産生され得る。

【００５５】

好ましくは、本発明のｓｉＲＮＡは、保護リボヌクレオシドホスホラミダイト、及び従来のＤＮＡ／ＲＮＡシンセサイザーを適切に用いて化学的に合成されるものである。ｓｉＲＮＡは、２つ分離した相補的なＲＮＡ分子として、若しくは２つの相補的領域を有する１つのＲＮＡ分子として合成され得る。合成ＲＮＡ分子若しくは合成試薬の商業的供給元としては、Ｐｒｏｌｉｇｏ（Ｈａｍｂｕｒｇ，ドイツ）、Ｄｈａｒｍａｃｏｎ Ｒｅｓｅａｒｃｈ（Ｌａｆａｙｅｔｔｅ，コロラド州，アメリカ），Ｐｉｅｒｃｅ Ｃｈｅｍｉｃａｌ（Ｐｅｒｂｉｏ Ｓｃｉｅｎｃｅの一部，Ｒｏｃｋｆｏｒｄ，イリノイ州，アメリカ），Ｇｌｅｎ Ｒｅｓｅａｒｃｈ（Ｓｔｅｒｌｉｎｇ，ヴァージニア州，アメリカ），ＣｈｅｍＧｅｎｅｓ（Ａｓｈｌａｎｄ，マサチューセッツ州，アメリカ），及びＣｒｕａｃｈｅｍ（Ｇｌａｓｇｏｗ，英国）を含む。

【００５６】

あるいは、ｓｉＲＮＡは、あらゆる適切なプロモーターを用いて組換え環状ＤＮＡプラスミド、若しくは直線ＤＮＡプラスミドからも発現され得る。プラスミドからの本発明のｓｉＲＮＡを発現するための適切なプロモーターは、例えば、Ｕ６若しくはＨ１ＲＮＡ ｐｏｌ ＩＩＩプロモーター配列，若しくはサイトメガロウイルスプロモーターを含む。他の適切なプロモーターの選択は、当業者の技術範囲内である。本発明の前記組換えプラスミドは、特定の組織或いは特定の細胞内環境におけるｓｉＲＮＡの発現のための誘導性プロモーター若しくは制御可能なプロモーターも有することができる。

【００５７】

組換えプラスミドから発現されたｓｉＲＮＡは、標準技術によって培養細胞発現システムから単離され得る、若しくは細胞内で発現され得る。本発明のｓｉＲＮＡを細胞へｉｎ ｖｉｖｏで運搬するための組換えプラスミドの使用は、以下でより詳細に論じる。

【００５８】

本発明のｓｉＲＮＡは、２つの分離した相補的なＲＮＡ分子として、若しくは２つの相補的な領域を有する１つのＲＮＡ分子として、組換えプラスミドから発現され得る。

【００５９】

本発明のｓｉＲＮＡを発現するのに適したプラスミドの選択、前記ｓｉＲＮＡを発現するためにプラスミドへ核酸配列を挿入する方法、及び、目的細胞へ組換えプラスミドを運搬する方法は、当業者の技術範囲内である。例えば、Ｔｕｓｃｈｌ，Ｔ．（２００２），Ｎａｔ．Ｂｉｏｔｅｃｈｎｏｌ，２０：４４６～４４８；Ｂｒｕｍｍｅｌｋａｍｐ ＴＲ ｅｔ ａｌ．（２００２），Ｓｃｉｅｎｃｅ ２９６：５５０～５５３；ＭｉｙａｇｉｓｈｉＭ ｅｔ ａｌ．（２００２），Ｎａｔ．Ｂｉｏｔｅｃｈｈｎｏｌ．２０：４９７～５００；Ｐａｄｄｉｓｏｎ ＰＪ ｅｔ ａｌ．（２００２），Ｇｅｎｅｓ Ｄｅｖ．１６：９４８～９５８；Ｌｅｅ ＮＳ ｅｔ ａｌ．（２００２），Ｎａｔ．Ｂｉｏｔｅｃｈｎｏｌ．２０：５００～５０５；Ｐａｕｌ ＣＰ ｅｔ ａｌ．（２００２），Ｎａｔ．Ｂｉｏｔｅｃｈｎｏｌ．２０：５０５～５０８を参照のこと（これらに開示された全体は、この参照により本明細書に組み込まれる）。

【００６０】

１実施形態において、本発明のｓｉＲＮＡを発現するプラスミドは、ヒトＵ６ ＲＮＡプロモーターの制御下でのｐｏｌｙＴ終止配列との操作可能な連結を持つセンスＲＮＡ鎖コード（翻訳）領域と、ヒトＵ６ ＲＮＡプロモーターの制御下でのｐｏｌｙＴ終止配列との操作可能な連結を持つアンチセンスＲＮＡ鎖コード領域とを有する。そのようなプラスミドは、本発明のｓｉＲＮＡを発現するための組換えアデノ随伴ウイルスベクターを産生する場合に用いられ得る。

【００６１】

ここで用いられたように、"ｐｏｌｙＴ終止配列との操作可能な連結"とは、センス鎖若しくはアンチセンス鎖をコード化する核酸配列が５’方向のｐｏｌｙＴ終止シグナルと即座に隣接することを意味する。前記プラスミドからの前記センス鎖若しくはアンチセンス鎖の転写の間、前記ｐｏｌｙＴ終止シグナルは転写を終了する働きをする。

【0062】

ここで用いられたように、プロモーターの"制御下"とは、前記プロモーターがセンス若しくはアンチセンスコード配列の転写を開始することができるように、前記センス鎖若しくはアンチセンス鎖をコード化する核酸配列がプロモーターの３’に位置することを意味する。

【0063】

本発明のｓｉＲＮＡは、ｉｎ　ｖｉｖｏ細胞内で組換えウイルスベクターからも発現され得る。本発明の組換えウイルスは、本発明のｓｉＲＮＡをコード化する配列と、ｓｉＲＮＡ配列を発現するための適切な任意のプロモーターとを有する。適切なプロモーターは、例えば、Ｕ６或いはＨ１　ｐｏｌＩＩＩプロモーター配列、及びサイトメガロウイルスプロモーターを含む。他の適切なプロモーターの選択は、当業者の技術範囲内である。本発明の組換えウイルスベクターは、特定の組織若しくは特定の細胞内環境においてｓｉＲＮＡを発現するための誘導性若しくは制御可能なプロモーターも有することができる。本発明のｓｉＲＮＡをｉｎ　ｖｉｖｏで細胞へ運搬するための組換えウイルスベクターの使用は、以下により詳細に述べる。

【0064】

本発明のｓｉＲＮＡは、２つの分離した相補的なＲＮＡ分子として、若しくは２つの相補的な領域を持つ１つのＲＮＡ分子として、組換えウイルスベクターから発現され得る。

【0065】

ｓｉＲＮＡ分子を発現するためのコード配列を受容する能力があるあらゆるウイルスベクターで用いられ得、例えば、アデノウイルス（ＡＶ）；アデノウイルス随伴ウイルス（ＡＡＶ）；レトロウイルス（例えばレンチウイルス（ＬＶ）、ラブドウイルス、マウス白血病ウイルス）；ヘルペスウイルス、及びそれと同等なものから由来したベクターなどである。ウイルスベクターの指向性は、ベクターをエンベロープタンパク質や他のウイルスからの別の表面抗原によってシュードタイピングすることによって、若しくは異なるウイルスキャプシドタンパク質で置換することによって、適切に修飾され得る。

【0066】

例えば、本発明のレンチウイルスベクターは、水疱性口内炎ウイルス（ＶＳＶ）、狂犬病ウイルス、エボラウイルス、　Ｍｏｋｏｌａウイルス、およびそれと同等なものからの表面タンパク質でシュードタイピングされ得る。本発明のＡＡＶベクターは、異なるキャプシドタンパク質血清型（ｓｅｒｏｔｙｐｅ）を発現するようにベクターを設計することによって、異なる細胞を標的として作成され得る。例えば、血清型２ゲノム上の血清型２キャプシドを発現するＡＡＶベクターは、ＡＡＶ２／２と呼ばれる。ＡＡＶ２／２ベクターにおけるこの血清型２キャプシド遺伝子は、ＡＡＶ２／５ベクターを産生するために、血清型５キャプシド遺伝子によって置換され得る。異なるキャプシドタンパク質血清型を発現するＡＡＶベクターを構成するための技術は、当業者の技術範囲内であり、例えば、Ｒａｂｉｎｏｗｉｔｚ　ＪＥ　ｅｔ　ａｌ．（２００２），Ｊ　Ｖｉｒｏｌ　７６：７９１～８０１を参照のこと（なお、この全体の開示は、この参照により本明細書に組み込まれる。）。

【0067】

好ましいウイルスベクターは、レンチウイルス、ＡＶ若しくはＡＡＶから由来したベクターである。特に好ましい実施形態において、本発明のｓｉＲＮＡは、例えば、Ｕ６或いはＨ１ＲＮＡプロモーター、若しくはサイトメガロウイルス（ＣＭＶ）プロモーターを有する組換えＡＡＶベクターから、２つの分離した相補的な一本鎖ＲＮＡ分子として発現される。本発明のｓｉＲＮＡを発現するための適切なＡＶベクター、組換えＡＶベクターを構成するための方法、及び前記ベクターを標的細胞へ運搬するための方法は、Ｘｉａ　Ｈ

ｅｔ　ａｌ．（２００２），Ｎａｔ．Ｂｉｏｔｅｃｈ．２０：１００６～１０１０に記載されている。

【００６８】

本発明のｓｉＲＮＡを発現するための適切なＡＡＶベクター、組換えＡＡＶベクターを構成するための方法、及び前記ベクターを標的細胞へ運搬する方法は、Ｓａｍｕｌｓｋｉ　Ｒ　ｅｔ　ａｌ．（１９８７），Ｊ．Ｖｉｒｏｌ．６１：３０９６～３１０１；Ｆｉｓｈｅｒ　ＫＪ　ｅｔ　ａｌ．（１９９６），Ｊ．Ｖｉｒｏｌ，７０：５２０～５３２；Ｓａｍｕｌｓｋｉ Ｒ　ｅｔ　ａｌ．（１９８９），Ｊ．Ｖｉｒｏｌ．６３：３８２２～３８２６；米国特許第５，１３９，９４１号公報；国際特許出願第ＷＯ９４／１３７８８号；国際特許出願第ＷＯ９３／２４６４１号に記載されており、これらの全体の開示は、この参照によって本明細書に組み込まれる。

【００６９】

標的ｍＲＮＡのＲＮＡｉ仲介性分解を引き起こすための所定の標的配列を含むｓｉＲＮＡの能力は、細胞中のＲＮＡレベル若しくはタンパク質レベルを測定するための標準技術を用いて評価され得る。例えば、本発明のｓｉＲＮＡは培養細胞へ運搬され得、標的ｍＲＮＡのレベルはノーザンブロット若しくはドットブロッティング技術によって、若しくは定量ＲＴ－ＰＣＲによって測定され得る。或いは、培養細胞中のＩＣＡＭ－１タンパク質のレベルは、ＥＬＩＳＡ若しくはウエスタンブロットによって測定され得る。

【００７０】

例えば、天然にＩＣＡＭ－１を発現している、若しくはＩＣＡＭ－１が発現するように誘導される細胞は、９６ウェルマイクロタイタープレート中でコンフルウエンス（ｃｏｎｆｌｕｅｎｃｅ）まで増殖される。天然にＩＣＡＭ－１を発現している細胞に対して、この細胞は、ＩＣＡＭ－１発現を刺激するために、インターロイキン－１若しくは腫瘍壊死因子で８～２４時間、刺激され得る。本発明のｓｉＲＮＡは、第１群のＩＣＡＭ－１発現細胞に投与される。非特異的ｓｉＲＮＡ（若しくはｓｉＲＮＡなし）は、対照として、第２群のＩＣＡＭ－１発現細胞に投与される。細胞は洗浄され、１～２％パラホルムアルデヒドでマイクロタイタープレートのウェルに直接固定された。マイクロタイタープレート上の非特異結合部位は、２％ウシ血清アルブミンでブロックされ、細胞はＩＣＡＭ－１特異的モノクローナル抗体とインキュベートされた。ＩＣＡＭ－１抗体の結合は、例えば、１：１０００希釈のビオチン標識ヤギ抗マウスＩｇＧ（Ｂｅｔｈｅｓｄａ　Ｒｅｓｅａｒｃｈ　Ｌａｂｏｒａｔｏｒｉｅｓ，Ｇａｉｔｈｅｒｓｂｅｒｇ，Ｍｄ）と１時間、３７℃でインキュベーションし、１：１０００希釈のベーターガラクトシダーゼと共役したストレプトアビジン（Ｂｅｔｈｅｓｄａ　Ｒｅｓｅａｒｃｈ　Ｌａｂｏｒａｔｏｒｉｅｓ）と１時間３７℃でインキュベーションすることによって、検出される。ＩＣＡＭ－１特異的モノクローナル抗体と結合したベーターガラクトシダーゼは、例えば、３．３ｍＭクロロフェノールレッド－ベーターＤ－ガラクトピラノシド、５０ｍＭリン酸ナトリウム、１．５ｍＭ　$MgCl_2$（ｐＨ７．２）の溶液で、２～１５分、３７℃でマイクロタイタープレートを展開し、ＥＬＩＳＡマイクロタイタープレートリーダーを用いて５７５ｎｍで結合した抗体の濃度を測定することによって決定される。

【００７１】

ＩＣＡＭ－１発現を下方制御する本発明のｓｉＲＮＡの能力は、神経突起伸長、内皮細胞間の接着、上皮細胞間の接着（例えば、正常ラット腎臓細胞及び／若しくはヒト皮膚）、若しくは癌細胞間の接着を当業者の技術範囲内の技術によって測定することによって、ｉｎ　ｖｉｔｒｏでも評価され得る。

【００７２】

適切な神経突起伸長アッセイは、ＩＣＡＭ－１を天然に発現している、若しくはＩＣＡＭ－１の発現を誘導されている単層細胞上の培養ニューロンを有する。ＩＣＡＭ－１発現細胞上のニューロン伸長は、ＩＣＡＭ－１が発現していない細胞上で培養されたニューロンに比べて、長く突起が伸長していた。例えば、ニューロンは、ＩＣＡＭ－１をコードしているｃＤＮＡで形質移入された３Ｔ３細胞の単層上に培養され得、基本的には、Ｄｏｈ

ｅｒｔｙ　ａｎｄ　Ｗａｌｓｈ，１９９４，Ｃｕｒｒ．Ｏｐ．Ｎｅｕｒｏｂｉｏｌ．４：４９～５５、及びＳａｆｅｌｌ　ｅｔ　ａｌ．，１９９７、Ｎｅｕｒｏｎ　１８：２３１～２４２に記載された通りであり、これらに開示された全体はこの参照によって本明細書に組み込まれる。簡潔に説明すると、対照３Ｔ３線維芽細胞、及びＩＣＡＭ－１を発現した３Ｔ３線維芽細胞の単層は、８ウェル組織培養ディッシュの各ウェルに８０，０００細胞を一晩培養することによって、達成される。出生前３日目のマウス脳から単離された３０００個の小脳ニューロンは、様々な単層上で１８時間培養される。次にその培養は固定され、標準技術を用いてニューロン特異的抗体（例えばＧＡＰ４３）で染色される。対照細胞及び本発明のｓｉＲＮＡで処理された細胞の神経突起の長さは、コンピュータ補助形態計測によって測定される。テスト細胞中のＩＣＡＭ－１　ｍＲＮＡのｓｉＲＮＡ誘導性ＲＮＡｉ分解は、対照細胞と比較して少なくとも５０％、平均神経突起の長さが増加することによって示される。

【0073】

本発明のｓｉＲＮＡによるＩＣＡＭ－１のＲＮＡｉ分解は、細胞接着の離開を検出することによっても評価され得る。例えば、ＩＣＡＭ－１を天然に発現している、若しくはＩＣＡＭ－１発現が誘導された細胞は、細胞接着が可能な標準条件下で播種される。本発明のｓｉＲＮＡを投与することによる細胞接着の離開は、細胞の他の細胞からの退縮を観察することによって、２４時間以内に視覚的に決定される。細胞接着の離開を検出するための適切なアッセイは、以下の通りである。ウシ肺動脈内皮細胞は、無菌除去（ａｂｌａｔｉｏｎ）と、０．１％コラゲナーゼ（タイプＩＩ；Ｗｏｒｔｈｉｎｇｔｏｎ　Ｅｎｚｙｍｅ，Ｆｒｅｅｈｏｌｄ、ニュージャージー州）での消化によって播種される。播種された細胞は、１０％ウシ胎児血清（ＦＣＳ）と、１％抗生剤－抗真菌剤が添加されたダルベッコ最小必須培地（ＤＭＥＭ）で、３７℃、７％　CO_2（空気中）において培養される。この培養は、トリプシン－ＥＤＴＡで週１回継代され、２０，０００細胞／cm^2で組織培養プラスチックへ播種される。培養コンフルエンス（ｃｏｎｆｌｕｅｎｃｙ）が達成された後の約３日後である１週間の培養後、細胞は本発明のｓｉＲＮＡ、若しくは対照非特異的ｓｉＲＮＡで処理（例えば３０分）される。この細胞は、ｓｉＲＮＡが投与されて２４時間以内に１％パラホルムアルデヒドで固定され、細胞接着の離開の程度を、細胞の他の細胞からの退縮を観察することによって決定された。

【0074】

本発明のｓｉＲＮＡによる標的ｍＲＮＡ仲介性分解は、例えば、未熟網膜症（”ＲＯＰ”）マウスモデル、脈絡叢新血管新生（”ＣＮＶ”）マウス及びラットモデルなどの、新血管新生の動物モデルでも評価され得る。例えば、ＣＮＶラットにおける新血管新生領域は、以下の実施例２にあるように、本発明のｓｉＲＮＡの投与前後で測定され得る。ｓｉＲＮＡの投与による新血管新生領域における減少は、ＩＣＭＡ－１　ｍＲＮＡの下方制御と細胞接着の離開とを意味する。ＩＣＡＭ－１ｍＲＮＡの下方制御と、細胞接着の離開とは、以下の、ストレプトゾトシン誘導糖尿病性網膜症ラットモデル（実施例３）、ＶＥＧＦ誘導性網膜血管透過性とｌｅｕｋｏｓｔａｓｉｓのラットモデル（実施例４）、及び角膜／輪部損傷による眼球新血管新生のラットモデル（実施例５）にも示されている。

【0075】

上述したように、本発明のｓｉＲＮＡは、ヒトＩＣＡＭ－１　ｍＲＮＡ、若しくはその選択的スプライシング体、変異体或いは同族体のＲＮＡｉ仲介性分解を標的にし、引き起こす。本発明のｓｉＲＮＡによる標的ｍＲＮＡの分解は、ＩＣＡＭ－１遺伝子からの機能遺伝子産物の産生を減少する。従って、本発明は、対象中のＩＣＡＭ－１の発現を阻害する方法を提供し、これは、標的ｍＲＮＡが分解されるように、本発明のｓｉＲＮＡの有効量を対象に投与する工程を有する。

【0076】

ＩＣＡＭ－１遺伝子の産物は細胞間接着若しくは細胞とＲＣＭとの接着に必要とされるので、本発明は、細胞接着若しくは細胞接着仲介性病態を患っている対象における細胞接着を阻害する方法も提供する。１実施形態において、ＩＣＡＭ－１仲介性細胞接着は血管

新生を開始し維持するために必要とされるので、本発明は、本発明のｓｉＲＮＡによる標的ｍＲＮＡのＲＮＡｉ仲介性分解によって対象における血管新生を阻害する方法を提供する。別の実施形態において、本発明は糖尿病Ｉ型から生じる合併症を患った対象を、本発明のｓｉＲＮＡによる標的ｍＲＮＡのＲＮＡｉ仲介性分解によって治療する方法を提供する。好ましくは、本発明の方法によって治療される糖尿病Ｉ型から生じた合併症としては、糖尿病性網膜症、糖尿病性神経障害、糖尿病性腎障害、及び大血管性疾患（動脈疾患、脳血管疾患、及び末梢血管疾患を含む）である。

【0077】

ここで用いられたように、"対象"とは、ヒト、若しくは非ヒト動物を含む。好ましくは、前記対象はヒトである。

【0078】

ここで用いられたように、ｓｉＲＮＡの"有効量"とは、標的ｍＲＮＡのＲＮＡｉ仲介性分解を引き起こすのに十分な量、若しくは対象における細胞接着若しくは細胞接着仲介性病態の開始若しくは進行を阻害するのに十分な量を指す。

【0079】

標的ｍＲＮＡのＲＮＡｉ仲介性分解は、上述したｍＲＮＡ若しくはタンパク質の単離及び定量するための標準技術を用いて、対象の細胞中の標的ｍＲＮＡレベル若しくはタンパク質レベルを測定することによって検出され得る。

【0080】

細胞接着若しくは細胞接着仲介性病態の阻害は、例えば、炎症、網膜症、神経障害、腎障害、若しくは対象が治療される疾患若しくは障害の特性を示す他の症状の程度を検出することによって、対象における病態の進行を測定することによっても評価され得る。

【0081】

例えば、血管新生の阻害は、本発明のｓｉＲＮＡで処理する前後の新血管新生領域のサイズを観察することによってなど、対象における病原性若しくは非病原性血管新生の進行を直接測定することによって評価され得る。新血管新生領域が同じか減少していた場合は、血管新生の阻害を意味している。対象における新血管新生領域のサイズを観察及び測定するための技術は、当業者の技術範囲内であり、例えば、脈絡膜新血管新生の領域が検眼鏡検査によって観察され得る。

【0082】

血管新生の阻害は、血管新生に関連した病的状態における変化若しくは回復の観察を通じて推測することも可能である。例えば、ＡＭＤにおいて、視覚消失の遅延、停止、若しくは回復は、脈絡膜における血管新生の阻害を意味している。腫瘍に対しては、腫瘍成長の遅延、停止、或いは逆転、若しくは腫瘍転移の停止は、腫瘍部位での或いは近くでの血管新生の阻害を意味している。非病原性血管新生の阻害は、例えば、本発明のｓｉＲＮＡの投与による脂肪消失若しくはコレステロールレベルでの減少など、からも推測され得る。

【0083】

本発明の方法は、非病原性である血管新生、すなわち、対象における正常過程に起因する血管新生、を阻害するために用いられ得る。非病原性血管新生の例えとしては、子宮内膜新血管新生、及び脂肪組織或いはコレステロールの産生に関連した過程を含む。従って、本発明は、例えば、体重を調節する或いは脂肪消失を促進するための、コレステロールレベルを減少するための、若しくは堕胎薬としての、非病原性血管新生を阻害するための方法も提供する。

【0084】

本発明の方法は、血管新生病、すなわち、不適切な或いは非制御の血管新生に関連した病原性の病気、に関連した血管新生も阻害することができる。例えば、ほとんどの悪性固形腫瘍は、腫瘍内と周囲に血管新生を誘導することによって腫瘍自体に十分な血液供給を発生する。この腫瘍誘導性血管新生は、腫瘍成長に対してしばしば必要とされ、転移性細胞が血流に入ることを可能にする。

【0085】
他の血管新生病としては、糖尿病性網膜症及び年齢関連性黄斑変性症（ＡＭＤ）を含む。これらの病気は、新血管新生の領域における新しく形成された血管による正常組織の破壊によって特徴付けられている。例えば、ＡＭＤにおいて、脈絡膜は、毛細血管によって侵入され、破壊されている。ＡＭＤにおける脈絡膜の血管新生に由来する破壊は、最終的には部分的な或いは完全な失明を導く。
【0086】
好ましくは、本発明のｓｉＲＮＡは、癌、例えば乳癌、肺癌、頭部と頸部の癌、膠腫、腹腔内腫瘍、大腸癌、結腸直腸癌、食道癌、胃腸癌、グリオーマ、肝癌、舌癌、神経芽細胞腫、卵巣癌、膵臓癌、前立腺癌、網膜芽細胞腫、ウィルムス腫瘍、多発性骨髄腫、皮膚癌（例えば黒色腫）、リンパ腫、及び血液癌などに関連した固形腫瘍の成長若しくは転移を阻害するために用いられる。
【0087】
より好ましくは、本発明のｓｉＲＮＡは、糖尿病Ｉ型から生じる合併症、例えば、糖尿病性網膜症、糖尿病性神経障害、糖尿病性腎障害、及び大血管性疾患など、を治療するために用いられる。
【0088】
細胞接着若しくは細胞接着仲介性病態を治療するために、特に、血管新生病及び糖尿病Ｉ型から生じた合併症を治療するために、本発明のｓｉＲＮＡは、本発明のｓｉＲＮＡとは異なる薬剤との組み合わせで対象に投与され得る。或いは、本発明のｓｉＲＮＡは、前記病態を治療するために設計された別の治療方法との組み合わせで対象に投与され得る。例えば、本発明のｓｉＲＮＡは、癌を治療する、若しくは腫瘍転移を予防するために現在用いられる医療方法（例えば、放射線療法、化学療法、及び手術）との組み合わせで投与され得る。腫瘍を治療するために、本発明のｓｉＲＮＡは好ましくは、放射線療法との組み合わせで、若しくはシスプラチン、カルボプラチン、シクロホスファミド、５－フルオロウラシル、アドリアマイシン、ダウノルビシン、若しくはタモキシフェンなどの化学療法薬剤との組み合わせで、投与される。
【0089】
本発明のｓｉＲＮＡは、サブ化学量論的な量でＲＮＡ干渉（従って、細胞接着を阻害する）を仲介することができることが理解される。どんな理論に束縛されることなく、本発明のｓｉＲＮＡは、ＲＩＳＣを誘導し、触媒様態で標的ｍＲＮＡを分解すること考えられている。従って、細胞接着若しくは細胞接着仲介性病態に対する標準的な治療法と比較して、治療的効果を発揮するように対象に投与される必要があるｓｉＲＮＡは、著しく少ない。
【0090】
当業者は、例えば、対象のサイズや体重、疾患侵食度の程度（長さ）、年齢、対象の健康度と性別、投与経路、及び投与が局所的なのか全身的なのか、などの評価要素を配慮することによって、特定の対象に投与される本発明のｓｉＲＮＡの有効量を容易に決定することができる。一般的に、本発明のｓｉＲＮＡの有効量は、細胞間若しくは細胞－マトリックス接着が阻害される部位での細胞内濃度が、約１ナノモル（ｎＭ）～約１００ｎＭ、好ましくは約２ｎＭ～５０ｎＭ、より好ましくは約２．５ｎＭ～約１０ｎＭである。
【0091】
本発明の方法において、本発明のｓｉＲＮＡは、運搬試薬との結合における裸の（ｎａｋｅｄ）ｓｉＲＮＡとして、若しくはｓｉＲＮＡを発現する組換えプラスミド或いはウイルスベクターとして、対象に投与され得る。
【0092】
本発明のｓｉＲＮＡとの結合での投与に対する最適な運搬試薬は、ｔｈｅ　Ｍｉｒｕｓ　Ｔｒａｎｓｉｔ　ＴＫＯ　ｌｉｐｏｐｈｉｌｉｃ　ｒｅａｇｅｎｔ（遷移ＴＫＯ親油性試薬）、リポフェクチン、リポフェクタミン、セルフェクチン、若しくはポリカチオン（例えば、ポリリジン）或いはリポソームを含む。好ましい運搬試薬は、リポソームである

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【００９３】
リポソームは、例えば、細胞若しくは腫瘍組織などの特定の組織へのｓｉＲＮＡの運搬を助け、ｓｉＲＮＡの血中半減期も増加することができる。本発明での使用に適したリポソームは、一般的に中性若しくは負電荷リン脂質及びコレステロールなどのステロールを含む、標準ベシクル（小胞）形成脂質から形成される。リポソームを準備するための様々な方法が知られており。例えば、Ｓｚｏｋａ　ｅｔ　ａｌ．（１９８０），Ａｎｎ．Ｒｅｖ．Ｂｉｏｐｈｙｓ．Ｂｉｏｅｎｇ．９：４６７、及び米国特許第４，２３５，８７１号、第４，５０１，７２８号、第４，８３７，０２８号、及び第５，０１９，３６９号公報があり、これら全体の開示はこの参照によって本発明に組み込まれる。
【００９４】
好ましくは、本発明のｓｉＲＮＡを被包するリポソームはリガンド分子を有しており、このリガンド分子は、血管新生部位、若しくは腫瘍などのＩＣＡＭ－１仲介性細胞接着が関連した他の病的過程の部位で或いは近くで、ＩＣＡＭ－１を発現する細胞に対するリポソームを標的とするものである。ＩＣＡＭ－１を発現する細胞は、内皮細胞、上皮細胞、線維芽細胞、造血細胞、及び腫瘍細胞を含む。腫瘍抗原若しくは内皮細胞表面抗原において広く見られる受容体と結合するリガンドは好ましい。
【００９５】
特に好ましくは、本発明のｓｉＲＮＡを被包しているリポソームは、例えば、構造の表面に結合するオプソニン化阻害部位を有することによって、単核マクロファージや網膜内皮性システムによるクリアランス（排除）を避けるように修飾される。１実施形態において、本発明のリポソームは、オプソニン化阻害部位、及びリガンドの両方を有することができる。
【００９６】
本発明のリポソームを準備するために用いられるオプソニン化阻害部位は、リポソーム膜に結合する典型的に大きな親水性ポリマーである。ここで用いられたように、オプソニン化阻害部位は、化学的に或いは物理的にリポソーム膜に結合する場合、例えば、膜自体への脂質－可溶性アンカーの挿入によって、若しくは、膜脂質の活性基への直接的な結合によって、リポソーム膜へ"結合"されるものである。これらのオプソニン化阻害親水性ポリマーは、マクロファージ－単球システム（"ＭＭＳ"）及び網膜内皮性システム（"ＲＥＳ"）によるリポソームの取り込みが著しく減少した保護表面層を形成し、例えば、米国特許第４，９２０，０１６号公報に記載されており、これに開示された全体はこの参照によって本発明に組み込まれる。従って、オプソニン化阻害部位で修飾されたリポソームは、非修飾リポソームに比べて、より長く循環中に残る。この理由により、そのようなリポソームはしばしば"ステルス（ｓｔｅａｌｔｈ；捕捉されない）"リポソームと呼ばれる。
【００９７】
ステルスリポソームは、多孔性或いは"漏出性"微小血管によって供給された組織内に蓄積されることが知られている。従って、そのような微小血管欠損によって特徴付けられた組織、例えば固形腫瘍などは、これらのリポソームが効率的に蓄積する（Ｇａｂｉｚｏｎ，ｅｔ　ａｌ．（１９８８），Ｐ．Ｎ．Ａ．Ｓ．，ＵＳＡ，１８：６９４９～５３を参照のこと）。加えて、ＲＥＳによる減少した取り込みは、肝臓及び脾臓における蓄積を著しく阻止することによって、ステルスリポソームの毒性を低くする。従って、オプソニン化阻害部位で修飾された本発明のリポソームは、本発明のｓｉＲＮＡを腫瘍細胞へ運搬するのに特に適している。
【００９８】
リポソームを修飾するのに適しているオプソニン化阻害部位は、好ましくは、平均分子量が約５００～約４０，０００ダルトンの水可溶性ポリマーであり、より好ましくは約２，０００～約２０，０００ダルトンの水可溶性ポリマーである。そのようなポリマーは、ポリエチレングリコール（ＰＥＧ）、若しくはポリプロピレングリコール（ＰＰＧ）誘導体（例えばメトキシＰＥＧ或いはＰＰＧ、及びＰＥＧ或いはＰＰＧステアリン酸）、ポリ

アクリルアミド或いはポリＮ－ビニルピロリドンなどの合成ポリマー、直線状、分枝状、或いは樹状（ｄｅｎｄｒｉｍｅｒｉｃ）ポリアミドアミン、ポリアクリル酸、ポリアルコール（例えば、カルボキシル基或いはアミノ基が化学的に結合したポリビニルアルコール及びポリキシリトール）、及びガングリオシドＧＭ$_1$などのガングリオシド、を含む。ＰＥＧのコポリマー、メトキシＰＥＧ或いはメトキシＰＰＧ、若しくはそれらの誘導体も適している。加えて、前記オプソニン化阻害ポリマーは、ＰＥＧのブロックコポリマー、及びポリアミノ酸、ポリサッカライド、ポリアミドアミン、ポリエチレンアミン、若しくはポリヌクレオチドも含むことができる。前記オプソニン化阻害ポリマーは、例えばガラクツロン酸、グルクロン酸、マンヌロン酸、ヒアルロン酸、ペクチン酸、ノイラミン酸、アルギン酸、カラギーナン、アミノ化ポリサッカライド或いはオリゴサッカライド（直線状或いは分枝状）などのアミノ酸若しくはカルボキシル酸、若しくは、例えばカルボキシル基の結合の原因となるカルボン酸の誘導体と反応した、カルボキル化ポリサッカライド或いはオリゴサッカライド、を含む天然ポリサッカライドも含むことができる。

【0099】

好ましくは、オプソニン化阻害部位は、ＰＥＧ、ＰＰＧ、若しくはそれらの誘導体である。ＰＥＧ若しくはＰＥＧ誘導体で修飾されたリポソームは、しばしば"ＰＥＧ化（ＰＥＧｙｌａｔｅｄ）リポソーム"と呼ばれる。

【0100】

オプソニン化阻害部位は、多数の既知技術における任意の一つによってリポソーム膜と結合可能である。例えば、ＰＥＧのＮ－ヒドロキシスクシニミドエステルは、ホスファチジル－エタノールアミン脂溶性アンカーと結合し、次に膜と結合することができる。同様に、デキストランポリマーは、Ｎａ（ＣＮ）ＢＨ３、及び例えばテトロヒドロフランと水（３０：１２の割合）などの溶媒混合液を６０℃で用いて、還元性アミノ化を介してステアリルアミン脂溶性アンカーで誘導体化され得る。

【0101】

本発明のｓｉＲＮＡを発現する組換えプラスミドは、上記で説明された。そのような組換えプラスミドは、直接若しくは、ｔｈｅ Ｍｉｒｕｓ Ｔｒａｎｓｉｔ ＬＴ１ ｌｉｐｏｐｈｉｌｉｃ ｒｅａｇｅｎｔ（遷移ＬＴ１組油性試薬）、リボフェクチン、リボフェクタミン、セルフェクチン、若しくはポリカチオン（例えば、ポリリジン）或いはリポソームを含む適切な運搬試薬との組み合わせて投与され得る。本発明のｓｉＲＮＡを発現する組換えウイルスベクターも、上記で説明されており、ＩＣＡＭ－１を発現している対象の細胞へそのようなベクターを運搬するための方法は、当業者の技術範囲内である。

【0102】

本発明のｓｉＲＮＡは、ＩＣＡＭ－１を発現する細胞へ前記ｓｉＲＮＡを運搬するのに適したあらゆる手段によって対象へ投与され得る。ＩＣＡＭ－１を発現している細胞は、例えば、血管内皮細胞、胸腺及び他の上皮細胞、及び線維芽細胞などの非造血性細胞、及び組織マクロファージ、分裂促進因子で刺激されたＴリンパ球芽細胞、及び扁桃腺、リンパ節、パイエル板における胚中心樹状細胞などの造血性細胞を含む。当業者は、特定の細胞は特定の状態でＩＣＡＭ－１を発現する、例えば、ＩＣＡＭ－１は、糖尿病性網膜症或いはＡＭＤなどの眼球新生血管疾患における網膜血管内皮細胞で発現することを理解する。

【0103】

本発明のｓｉＲＮＡをＩＣＡＭ－１発現細胞へ運搬するのに適した技術としては、ジーンガン、電気穿孔法、ナノ粒子、マイクロ被包、及びそれらと同等のものによって、若しくは非経口及び経腸の投与経路によって、対象へ前記ｓｉＲＮＡを投与する方法を含む。

【0104】

適した経腸投与経路は、経口、直腸、若しくは鼻腔内運搬を含む。

【0105】

適した非経口投与経路は、血管内投与（例えば、静脈内大量瞬時投与、静脈内注入、動脈内大量瞬時投与、動脈内注入、及び脈管構造へのカテーテル滴下など）、組織周囲およ

び組織内投与（例えば、腫瘍周囲及び腫瘍内注射、網膜内注射、若しくは網膜下注射など）、皮下注射、或いは（浸透圧ポンプなどによる）皮下注入を含む沈着、例えば、カテーテル或いは他の設置装置（例えば角膜ペレット或いは坐薬、目薬、若しくは多孔性、非多孔性、或いはゼラチン物質を有する移植片）による新生血管新生の領域或いは部位の周辺への直接（例えば局所的）投与、及び吸入、を含む。適した設置装置は、米国特許第５，９０２，５９８号及び第６，３７５，９７２号公報に記載された眼球移植片、及び米国特許第６，３３１，３１３号公報に記載された生分解性眼球移植片を含む。また、これら特許文献に開示された全体は、この参照により本明細書に組み込まれる。そのような眼球移植片は、Ｃｏｎｔｒｏｌ　Ｄｅｌｉｖｅｒｙ　Ｓｙｓｔｅｍｓ，Ｉｎｃ．（Ｗａｔｅｒｔｏｗｎ、マサチューセッツ州）、及びＯｃｕｌｅｘ　Ｐｈａｒｍａｃｅｕｔｉｃａｌｓ，Ｉｎｃ．（Ｓｕｎｎｙｖａｌｅ、カリフォルニア州）から利用可能である。

【0106】

好ましい実施形態において、前記ｓｉＲＮＡの注射若しくは注入は、新生血管新生の部位に若しくは近くになされる。例えば、本発明のｓｉＲＮＡは、眼の網膜色素上皮細胞へ運搬され得る。好ましくは、前記ｓｉＲＮＡは局所的に眼へ、例えば、液体若しくはゲル状で下目瞼或いは結膜円蓋へ、投与され、これは当業者の技術範囲内である（Ａｃｈｅａｍｐｏｎｇ　ＡＡ　ｅｔ　ａｌ．，２００２，Ｄｒｕｇ　Ｍｅｔａｂｏｌ．ａｎｄ　Ｄｉｓｐｏｓｉｔｉｏｎ　３０：４２１～４２９を参照のこと。またこの全体の開示はこの参照により本明細書に組み込まれる）。

【0107】

一般的に、本発明のｓｉＲＮＡは、約５μｌ～約７５μｌの量で、例えば約７μｌ～約５０μｌで、好ましくは約１０μｌ～約３０μｌの量で、眼へ局所的に投与される。本発明のｓｉＲＮＡは、水溶液に高い溶解性を有しており、７５μｌ以上の量でのｓｉＲＮＡの眼への局所的滴下は、流出や排液による眼からのｓｉＲＮＡの損失に終わることになる。従って、高濃度のｓｉＲＮＡ（例えば、約１０～約２００ｍｇ／ｍｌ、若しくは約１００～約１０００ｎＭなど）を約５μｌ～約７５μｌの量で眼へ局所滴下することによって投与することが好ましい。

【0108】

特に好ましい非経口投与経路は、眼内投与である。本発明のｓｉＲＮＡの眼内投与は、この投与経路によって前記ｓｉＲＮＡが眼に入ることが可能である限り、眼への注射若しくは直接（例えば局所的）投与によって達成され得る。上記の眼への局所的経路の投与に加えて、適した眼内投与経路としては、硝子体内、網膜内、網膜下、テノン嚢下（ｓｕｂｔｅｎｏｎ）、眼窩周辺及び眼窩後、トランス角膜及びトランス強膜投与を含む。そのような眼内投与経路は、当業者の技術範囲であり、例えば、Ａｃｈｅａｍｐｏｎｇ　ＡＡ　ｅｔ　ａｌ．，２００２、ｓｕｐｒａ、Ｂｅｎｎｅｔｔ　ｅｔ　ａｌ．（１９９６），Ｈｕｍ．Ｇｅｎｅ　Ｔｈｅｒ．７：１７６３～１７６９、及びＡｍｂａｔｉ　Ｊ　ｅｔ　ａｌ．，２００２，Ｐｒｏｇｒｅｓｓ　ｉｎ　Ｒｅｔｉｎａｌ　ａｎｄ　Ｅｙｅ　Ｒｅｓ．２１：１４５～１５１を参照のこと（全体の開示はこの参照により本明細書に組み込まれる）。

【0109】

本発明のｓｉＲＮＡは、単一用量（ｓｉｎｇｌｅ　ｄｏｓｅ）若しくは複数用量（ｍｕｌｔｉｐｌｅ　ｄｏｓｅ）で投与され得る。本発明のｓｉＲＮＡを注入で投与する場合、この注入は単一持続性用量である、若しくは複数注射（ｍｕｌｔｉｐｌｅ　ｉｎｊｅｃｔｉｏｎ）によって運搬され得る。組織への直接的な前記ｓｉＲＮＡの注射は、新生血管新生の部位若しくはその近くであることが好ましい。新生血管新生の部位若しくはその近くの組織への前記ｓｉＲＮＡの複数注射は、特に好ましい。

【0110】

当業者は、本発明のｓｉＲＮＡを所定の対象へ投与するための適切な用量処方を容易に決定することもできる。例えば、前記ｓｉＲＮＡは、単一注射若しくは新生血管新生部位へ若しくはその近くへの沈着など、対象へ一度で投与され得る。或いは、前記ｓｉＲＮＡ

は、毎日若しくは週１回で複数回、対象へ投与され得る。例えば、前記ｓｉＲＮＡは、約３～約２８週間、より好ましくは約７～約１０週間の期間で、週１回対象へ投与され得る。好ましい用量処方において、前記ｓｉＲＮＡは、新生血管新生の部位へ若しくはその近くへ（例えば硝子体内へ）、週１回で７週間注射され得る。無期限の期間での本発明のｓｉＲＮＡの周期的な投与は、ｗｅｔ　ＡＭＤ若しくは糖尿病性網膜症などの慢性新生血管新生病を患った対象にとって必要となることが理解される。

【0111】

用量処方が複数投与である場合、対象に投与されるｓｉＲＮＡの有効量は、全体の用量処方を超えて投与されたｓｉＲＮＡの総量より成ることが理解される。

【0112】

本発明のｓｉＲＮＡは、本分野で知られた技術によって、対象に投与する前に薬学的組成物として処方されることが好ましい。本発明の薬学的組成物は、少なくとも無菌で発熱物質を含まないものとして特徴付けられる。ここで用いられる、”薬学的な処方”とは、ヒトへの及び獣医学的な使用に対する処方を含む。本発明の薬学的組成物を調合する方法は、当業者の技術範囲内であり、例えば、Ｒｅｍｉｎｇｔｏｎ’ｓ　Ｐｈａｒｍａｃｅｕｔｉｃａｌ　Ｓｃｉｅｎｃｅ，１７ｔｈ　ｅｄ，Ｍａｃｋ　Ｐｕｂｌｉｓｈｉｎｇ　Ｃｏｍｐａｎｙ，Ｅａｓｔｏｎ，Ｐａ．（１９８５）に記載されており、この全体の開示はこの参照により本明細書に組み込まれる。

【0113】

本発明の薬学的な処方は、生理学的に許容可能な担体培地と混合された、本発明のｓｉＲＮＡ（例えば、０．１～９０重量％）、若しくは生理学的に許容可能なその塩類を有する。好ましい生理学的に許容可能な担体培地は、水、緩衝液、食塩水（例えば、通常食塩水若しくはハンクス或いはアール平衡塩類溶液などの平衡塩類溶液）、０．４％食塩水、０．３％グリシン、ヒアルロン酸、及びそれらと同等のものである。

【0114】

本発明の薬学的組成物は、従来の薬学的賦形剤及び／若しくは添加剤も有する。適した薬学的賦形剤は、安定剤、抗酸化剤、重量モル浸透圧濃度調節因子、緩衝液、及びｐＨ調節因子を含む。適した添加剤は、生理学的な生体適合性緩衝液（例えば、トロメタミン塩酸塩）、キレートの添加（例えばＤＴＰＡ若しくはＤＴＰＡ－ビスアミドなど）或いはカルシウムキレート複合剤（例えばカルシウムＤＴＰＡ、ＣａＮａＤＴＰＡ－ビスアミドなど）、若しくは任意に、カルシウム或いはナトリウム塩の添加（例えば、塩化カルシウム、アスコルビン酸カルシウム、グルコン酸カルシウム、或いは乳酸カルシウムなど）を含む。本発明の薬学的組成物は、液状での使用のために調合（包装）される、若しくは凍結乾燥される。

【0115】

眼への局所的投与のために、従来の眼内運搬因子が用いられ得る。例えば、局所的眼内運搬のための本発明の薬学的組成物は、上述した食塩水、角膜浸透増進剤、不溶性粒子、ワセリン或いは他のゲル状軟膏、眼における滴下による粘性増加に耐えるポリマー、若しくは粘膜付着性ポリマーを有する。好ましくは、前記眼内運搬因子は、角膜浸透が増加する、若しくは粘性効果を通じて、或いは角膜上皮を覆っているムチン層との物理化学的な相互作用によって前記ｓｉＲＮＡの眼前（ｐｒｅｏｃｕｌａｒ）保持を延長する。

【0116】

局所的眼内運搬に適した不溶性粒子は、リン酸カルシウム粒子（Ｂｅｌｌらによる米国特許第６，３５５，２７１号公報に記載されており、この全体の開示はこの参照により本明細書に組み込まれる）。眼における滴下による粘性増加に耐える、適したポリマーは、ｐｏｌｏｘａｍｅｒ　４０７などのポリエチルエネポリオキシプロピレンブロックコポリマー（例えば２５％濃度）、セルロースアセトフタル酸（例えば、３０％濃度）、若しくはＧｅｌｒｉｔｅ（商標）（ＣＰ　Ｋｅｌｃｏ，Ｗｉｌｍｉｎｇｔｏｎ、デラウェア州）などの低アセチルゲラン（ｇｅｌｌａｎ）ガムを含む。適した粘膜付着性ポリマーは、カルボキシル、ヒドロキシル、アミド及び／若しくは硫酸基などの多数の親水性機能基を有

する親水コロイド（例えば、ヒドロキシプロピルセルロース、ポリアクリル酸、高分子量ポリエチレングリコール（例えば平均分子量が＞２００，０００）、デキストラン、ヒアルロン酸、ポリガラクツロン酸、及びキシロカン（ｘｙｌｏｃａｎ））を含む。適した角膜浸透増進剤は、シクロデキストリン、ベンザルコニウム塩化物、ポリオキシエチレングリコールラウリルエーテル（例えば、Ｂｒｉｊ（商標）３５）、ポリオキシエチレングリコールステアリルエーテル（例えば、Ｂｒｉｊ（商標）７８）、ポリオキシエチレングリコールオレイルエーテル（例えば、Ｂｒｉｊ（商標）９８）、エチレンジアミン四酢酸（ＥＤＴＡ）、ジギトニン、タウロコール酸ナトリウム、サポニン、及びＣｒｅｍａｐｈｏｒ　ＥＬなどのポリオキシエチル化ヒマシ油を含む。

【0117】

固体組成物のために、従来の無毒性固体単体が用いられ得、例えば、薬学的グレードのマンニトール、デンプン、ステアリン酸マグネシウム、サッカリンナトリウム、タルカム（滑石粉）、セルロース、グルコース、スクロース、マグネシウム炭酸塩、及びそれらと同等なものである。

【0118】

例えば、経口投与のための固体薬学的組成物は、上記の任意の担体と賦形剤、及び１０％～９５％、好ましくは２５％～７５％の１若しくはそれ以上の本発明のｓｉＲＮＡを有する。エアロゾル（吸入性）投与のための薬学的組成物は、０．０１重量％～２０重量％、好ましくは１重量％～１０重量％の１若しくはそれ以上の本発明のｓｉＲＮＡを有し、このｓｉＲＮＡは上述したようにリポソームに被包され、噴霧剤となる。例えば鼻腔内運搬のためのレシチンなどの担体も要望通りに含むことができる。

【0119】

本発明は、以下の限定されない実施例によって説明される。

【実施例】

【0120】

ヒトＩＣＡＭ－１　ｍＲＮＡを標的としたｓｉＲＮＡによるヒトＩＣＡＭ－１発現の阻害

サイトカイン若しくは低酸素によるＨＥＫ－２９３細胞中のＩＣＡＭ－１産生の刺激

ヒト胚性腎（ＨＥＫ）－２９３細胞におけるＩＣＡＭ－１の産生を刺激するための、低酸素若しくはサイトカインである腫瘍壊死因子アルファ（ＴＮＦ－α）やインターフェロンガンマ（ＩＦＮ－γ）の能力を評価した。

【0121】

ＨＥＫ－２９３細胞を、５％CO_2、３７℃で一晩、２４ウェルプレートにおいて標準成長培養液で培養した。次に細胞を、１、１０、１００或いは１０００ｎｇのＴＮＦ－α若しくはＩＦＮ－γ（Ｒ＆Ｄ　Ｓｙｓｔｅｍｓ、Ｍｉｎｎｅａｐｏｌｉｓ、ミネソタ州）で別々に処理した、若しくは、１００、２００或いは３００ｍＭデスフェリオキサミン（Ｓｉｇｍａ、Ｓｔ．Ｌｏｕｉｓ、ミズーリ州）での処理によって低酸素とした。サイトカイン若しくはデスフェリオキサミンでの処理の１或いは２日後、細胞をＭ－ＰＥＲ　Ｍａｍｍａｌｉａｎ　Ｐｒｏｔｅｉｎ　Ｅｘｔｒａｃｔｉｏｎ　ｒｅａｇｅｎｔ（哺乳類タンパク質抽出試薬）（Ｐｉｅｒｃｅ、Ｒｏｃｋｆｏｒｄ、イリノイ州）で溶解した。ヒトＩＣＡＭ－１　ＥＬＩＳＡ（Ｒ＆Ｄ　Ｓｙｓｔｅｍｓ、Ｍｉｎｎｅａｐｏｌｉｓ、ミネソタ州）を、Ｑｕａｎｔｉｋｉｎｅヒト"ｓＩＣＡＭ１"ＥＬＩＳＡ手順に記載されたように、細胞溶解液で実行し、このＥＬＩＳＡの結果はＡＤ３４０プレートリーダー（Ｂｅｃｋｍａｎ　Ｃｏｕｌｔｅｒ）で検出した。

【0122】

図１に示すように、ＨＥＫ－２９３細胞におけるヒトＩＣＡＭ－１タンパク質のレベルが上昇した唯一の条件は、１００ｎｇ／ｍｌ　ＴＮＦ－αで２日間細胞を処理したものであった。

【0123】

ヒトＩＣＡＭ－１　ｍＲＮＡを標的としたｓｉＲＮＡによる、刺激されたＨＥＫ－２９

３細胞の処理
ＨＥＫ－２９３細胞を、実施例１のように一晩培養した。細胞が約５０％コンフルエントになった時、実験細胞と対照細胞への形質移入を次の日実行した。実験細胞は、リン酸カルシウム形質転換試薬で混合した２５ｎＭヒトＩＣＡＭ－１　ｓｉＲＮＡで形質転換した。対照細胞は、ｓｉＲＮＡを欠いたリン酸カルシウム試薬で、若しくはリン酸カルシウム形質転換試薬中の２５ｎＭ非特異的ｓｉＲＮＡ（ＥＧＦＰ　ｓｉＲＮＡ）で処理した。
【0124】
実験細胞に対しては、ヒトＩＣＡＭ－１　ｍＲＮＡ内の異なる位置を標的とした１０個のｓｉＲＮＡを試験した。これらのｓｉＲＮＡは、表２に挙げた配列を標的としており、全てのｓｉＲＮＡは、３’ＴＴオーバーハングを各鎖に含んでいた。
【0125】
【表2】

Table 2－ＨＥＫ－２９３細胞でテストされたｓｉＲＮＡの標的配列

標的配列	配列ＩＤ番号：	siRNA
AATGCCCAGACATCTGTGTCC	20	hICAM1#1
AACAACCGGAAGGTGTATGAA	29	hICAM1#2
AACCGGAAGGTGTATGAACTG	30	hICAM1#3
AACCTTACCCTACGCTGCCAG	47	hICAM1#4
AACGACTCCTTCTCGGCCAAG	58	hICAM1#5
AACGTGATTCTGACGAAGCCA	62	hICAM1#6
AAGTGTGAGGCCCACCCTAGA	65	hICAM1#7
AACTGGACGTGGCCAGAAAAT	74	hICAM1#8
AAGTGTCTAAAGGATGGCACT	80	hICAM1#9
AACCGCCAGCGGAAGATCAAG	87	hICAM1#10

【0126】
形質転換４時間後、ＨＥＫ－２９３細胞中のＩＣＡＭ－１の産生は、最終濃度１００ｎｇ/ｍｌでのＴＮＦ－αで処理することによって刺激した。形質転換後４８時間で、全てのウェルから上清を除去した。実験細胞及び対照細胞の第１群は、Ｍ－ＰＥＲ　Ｍａｍｍａｌｉａｎ　Ｐｒｏｔｅｉｎ　Ｅｘｔｒａｃｔｉｏｎ　ｒｅａｇｅｎｔ（Ｐｉｅｒｃｅ、Ｒｏｃｋｆｏｒｄ、イリノイ州）で溶解した。ヒトＩＣＡＭ－１　ＥＬＩＳＡ（Ｒ&Ｄ　ｓｙｓｔｅｍｓ、Ｍｉｎｎｅａｐｏｌｉｓ、ミネソタ州）を、実施例１と同様に細胞溶解液で実行した。図２に示したように、ｈＩＣＡＭ１＃２、ｈＩＣＡＭ１＃３、ｈＩＣＡＭ１＃７、ｈＩＣＡＭ１＃９、及びｈＩＣＡＭ１＃１０のｓｉＲＮＡで刺激された、ＨＥＫ－２９３細胞中に誘導されたＩＣＡＭ－１タンパク質のレベルは、ｓｉＲＮＡなし、若しくは非特異的ｓｉＲＮＡで形質転換された細胞と比較して減少した。
【0127】
細胞毒性アッセイは、実験細胞及び対照細胞の第２群で実行した。上述したように形質転換後４８時間で上清を除去した後、１０％　ＡｌａｍａｒＢｌｕｅ（Ｂｉｏｓｏｕｒｃｅ、Ｃａｍａｒｉｌｌｏ、カナダ）を含む完全増殖培養液を、対象細胞及び実験細胞へと添加し、３７℃、５％　CO_2で3時間インキュベートした。細胞増殖は、細胞恒常性活性に起因する培養液の色変化を検出することによって測定した。この色変化は、ＡＤ３４０プレートリーダー（Ｂｅｃｋｍａｎ　Ｃｏｕｌｔｅｒ）で検出し、その結果は図３に示した。図３から分かるように、ｈＩＣＡＭ１＃１～９はＨＥＫ－２９３細胞における著し

い細胞毒性を示さなかった。ｈＩＣＡＭ１＃１０は、対照細胞と比較して、ＨＥＫ－２９３細胞増殖のわずかな減少を引き起こした。

【0128】

細胞毒性アッセイが実行された後、ＡｌａｍａｒＢｌｕｅを含む増殖培養液を完全に除去し、製造者説明書に従ってＲＮＡｑｕｅｏｕｓ ＲＮＡ ｉｓｏｌａｔｉｏｎ ｋｉｔ（ＲＮＡ単離キット）（Ａｍｂｉｏｎ、Ａｕｓｔｉｎ、テキサス州）を用いて、ＲＮＡをＨＥＫ－２９３細胞から抽出した。前記細胞中のヒトＩＣＡＭ－１　ｍＲＮＡのレベルは、定量逆転写ポリメラーゼ連鎖反応法（ＲＴ－ＰＣＲ）によって測定し、内部標準としてはヒトグリセルアルデヒド－３－ホスフェートデヒドロゲナーゼ（ＧＡＰＤＨ）ｍＲＮＡのレベルを用いた。このＲＴ－ＰＣＲ実験によって、ＴＮＦ－αによって誘導されたＩＣＡＭ－１の産生は、ｓｉＲＮＡなし若しくは非特異的ｓｉＲＮＡで形質転換された細胞と比較して、ヒトＩＣＡＭ－１　ｓｉＲＮＡによって、転写レベルで抑制された。

【実施例】

【0129】

ＩＣＡＭ－１を標的としたｓｉＲＮＡによるストレプトゾトシン誘導糖尿病性網膜症の治療

糖尿病性網膜症を患った個人の網膜における血管漏出と非灌流は、空間的、時間的に白血球静止と関連している。例えばＭｉｙａｍｏｔｏ Ｋ ｅｔ ａｌ．（１９９９），Ｐｒｏｃ．Ｎａｔ．Ａｃａｄ．Ｓｃｉ．ＵＳＡ９６（１９）：１０８３６～４１を参照し、この全体の開示はこの参照により本明細書に組み込まれる。ＩＣＡＭ－１を標的としたｓｉＲＮＡの硝子体内注射は、白血球静止を減少し、従って糖尿病ラットにおける網膜血管透過性を減少すると予想される。

【0130】

Ｍｉｙａｍｏｔｏ Ｋ ｅｔ ａｌ．（１９９９），ｓｕｐｒａ．に記載されたように、Ｌｏｎｇ－Ｅｖａｎｓ（ロングエバンス）ラット（約２００ｇ）に、一晩後、糖尿病を誘導するために、クエン酸緩衝液中のストレプトゾトシンを硝子体内に注射する。Ｌｏｎｇ－Ｅｖａｎｓラット（約２００ｇ）に、一晩後、対照としてクエン酸緩衝液のみを注射する。血清血液糖を測定し、血圧を毎日記録する。対照動物と比較しての血清血液糖レベルの上昇は、糖尿病であると考えられる。

【0131】

ＩＣＡＭ－１を標的としたｓｉＲＮＡの硝子体内注射は、各ラットにおいてＯＤを実行する。非特異的ｓｉＲＮＡは対照ＯＳとして注射される。全体の群図式は、表３に示している。

【0132】

【表3】

Table 3 – 全体の群図式

	OD (ICAM-1 siRNA)	OS (非特異的siRNA)
糖尿病ラット(STZ)	実験群	対照
非糖尿病ラット	対照	対照

【0133】

Ｍｉｙａｍｏｔｏ Ｋ ｅｔ ａｌ．（１９９９），ｓｕｐｒａ．に記載されたように、処理後７日目で、ラットをＡｃｒｉｄｉｎｅ Ｏｒａｎｇｅ Ｌｅｕｋｏｃｙｔｅ Ｆｌｕｏｒｏｇｒａｐｈｙ（アクリジンオレンジ白血球蛍光光度分析：ＡＯＬＦ）に供する。簡潔に言うと、ラットを麻酔し、その瞳孔をトロピカミドで散大した。次にラットに無

菌生理食塩水中に懸濁されたアクリジンオレンジを硝子体内に注射する。各眼の眼底を観察し、スキャニングレーザー眼底鏡（光源としてアルゴンブルーレーザー）を用いて白血球静止を画像化する。次にラットをフルオレセインデキストランで灌流し、眼をさらに画像化する。白血球静止の密度を、１０ディスク直径範囲における光ピクセルのパーセンテージとして計算する。この白血球静止の密度はエンドポイントとして用いられる。

【0134】

Ｍｉｙａｍｏｔｏ　Ｋ　ｅｔ　ａｌ．（１９９９），ｓｕｐｒａ．に記載されたように、７日目にラットはアイソトープ希釈技術にも供し、血管漏出を定量化する。簡潔に言うと、ラットに、第１の時点でＢＳＡ中のＩ１２５を、第２の時点でＩ１３１を硝子体内に注射する。このラットは第２の注射後、数分で屠殺し、その網膜を単離し、動脈サンプルを採取する。前記網膜及び前記動脈サンプルは、ヨウ素クリアランスの定量的指標を用いて網膜における活性を補正した後、γ一分光法を用いて解析する。次に測定値を、正確な用量を投与するために、体重及び組織重量を基に規準化する。γ活性の補正された量は、網膜内の血管漏出のマーカーとして用いる（第２エンドポイント）。実験動物の網膜内で減少するであろうγ活性は、血管漏出の減少を意味している。

【実施例】

【0135】

ＩＣＡＭ－１を標的としたｓｉＲＮＡによるＶＥＧＦ誘導血管透過性及び白血球静止の治療

眼内のＶＥＧＦの存在は、網膜内の血管透過性及び毛細血管非灌流の増加に対応する、網膜白血球静止を引き起こす。例えば、Ｍｉｙａｍｏｔｏ　ｅｔ　ａｌ．（２０００），Ａｍ．Ｊ．Ｐａｔｈｏｌ．１５６（５）：１７３３～９を参照し、この全体の開示はこの参照により本明細書に組み込まれる。ＩＣＡＭ－１を標的としたｓｉＲＮＡの硝子体内注射は、ラット内でＶＥＧＦの硝子体内注射によって作られた透過性及び白血球静止を減少すると予想される。

【0136】

Ｌｏｎｇ－Ｅｖａｎｓラット（約２００ｇ）に麻酔をし、緩衝液ＯＵ中のＶＥＧＦを硝子体内に注射する。ＩＣＡＭ－１を標的としたｓｉＲＮＡは、硝子体内注射によって各ラットへ同時にＯＤを運搬する。非特異的ｓｉＲＮＡは対照ＯＳとして硝子体内に注射する。付加的な対照は、緩衝液のみ（ＶＥＧＦなし）を注射されたラットを含む。全体の群図式は、表４に示している。

【0137】

【表4】

Table 4 – 全体の群図式

	OD (ICAM-1 siRNA)	OS (非特異的siRNA)
VEGF	実験群	対照
緩衝液	対照	対照

【0138】

注射後２４時間で、実施例１に記載したように、ラットをＡＯＬＦ、及びアイソトープ希釈技術に供する。

【実施例】

【0139】

ＩＣＡＭ－１を標的としたｓｉＲＮＡによる角膜／辺縁系損傷を患った眼における新血

管新生の治療
眼球表面の損傷は、角膜辺縁系幹細胞の破壊を生じる。これらの細胞の破壊は、ＶＥＧＦ依存性角膜新血管新生を誘導し、これは失明を導く。新血管新生を促進するＶＥＧＦは、好中球及び単球によって供給され、眼球表面に対する損傷の後、角膜を浸潤する。例えば、Ｍｏｒｏｍｉｚａｔｏ　Ｙ　ｅｔ　ａｌ．（２０００），Ａｍ．Ｊ．Ｐａｔｈｏｌ．１５７（４）：１２７７～８１を参照し、この全体の開示はこの参照により本明細書に組み込まれる。辺縁系損傷の後で角膜に適用される、ＩＣＡＭ－１を標的としたｓｉＲＮＡは、マウスにおける角膜の新血管新生の結果生じる領域を減少するであろうと予想される。新血管新生領域は、直接測定される、或いは、角膜新血管新生における減少は、角膜におけるＶＥＧＦ産生多形核細胞の数の減少から推測され得る。
【０１４０】
角膜新血管新生は、Ｍｏｒｏｍｉｚａｔｏ　Ｙ　ｅｔ　ａｌ．（ｓｕｐｒａ）で記載されたように、辺縁系の損傷によって、Ｃ５７Ｂｌ／６において誘導される。簡潔に言うと、マウスを麻酔し、水酸化ナトリウムを角膜へ適用する。前記角膜と辺縁系上皮は、角膜ナイフを用いて壊死組織切除した（ＯＵ）。ＩＣＡＭ－１を標的としたｓｉＲＮＡを、除去後すぐ、及び実験の間（７日間）１日３回、角膜表面ＯＤに適用した。非特異的ｓｉＲＮＡは、対照と同じ用量投与計画でＯＳに投与した。
【０１４１】
Ｍｏｒｏｍｉｚａｔｏ　Ｙ　ｅｔ　ａｌ．（２０００），ｓｕｐｒａ．に記載されたように、角膜及び辺縁系上皮の壊死組織切除後２、４、及び７日目に、マウスは角膜新血管新生の程度を評価する。簡潔に言うと、内皮特異的フルオレセイン共役レクチンを硝子体内に注射する。注射後３０分で、マウスを屠殺し、その眼を回収し、２４時間ホルマリンで固定する。角膜のフラットマウント（ｆｌａｔ　ｍｏｕｎｔｓ）を作成し、この角膜フラットマウントの写真を蛍光顕微鏡下で撮り、解析のためにＯｐｅｎｌａｂソフトウェアにインポートする。Ｏｐｅｎｌａｂソフトウェアを用いて、血管が見られる所でのみ蛍光の閾値レベルを設定する。蛍光血管の領域及び角膜の領域（辺縁系アーケードで境界を画定された）を計算する。血管の領域は、総角膜領域で分割され、この値は、新血管領域のパーセントと等しい。処置群及び対照群の新血管領域のパーセントを比較する。
【０１４２】
Ｍｏｒｏｍｉｚａｔｏ　Ｙ　ｅｔ　ａｌ．（２０００），ｓｕｐｒａ．に記載されたように、角膜及び辺縁系上皮の壊死組織切除後２、４、及び７日目に、付加的なマウスは、角膜多形核細胞（ＰＭＮｓ）の定量化のために屠殺する。簡潔に言うと、マウスを屠殺し、その眼を回収し、２４時間ホルマリンで固定する。ホルマリン固定の後、眼球除去された眼をパラフィンで包埋し、切片を作成した。角膜の解剖学的な中心と相関する、各眼からの１つのパラフィン切片を選択し、顕微鏡用に用いる。この１切片上のＰＭＮｓ（多小葉からなる細胞として同定される）を数え、処理群及び対照群からの切片中のＰＭＮｓの数を比較する。
【実施例】
【０１４３】
ＩＣＭＡ－１を標的としたｓｉＲＮＡによるレーザー誘導脈絡叢新血管新生の治療
Ｂｒｕｃｈ’ｓ膜を破裂するレーザー光凝固は、ウェット黄斑変性症において見られるものと同様な、脈絡叢新血管新生（ＣＮＶ）を誘導する。ＩＣＡＭ－１を標的としたｓｉＲＮＡの硝子体内注射は、マウスにおけるレーザー誘導ＣＮＶの領域を減少すると予想される。
【０１４４】
ＣＮＶはマウスにおいて、Ｓａｋｕｒａｉ　Ｅ　ｅｔ　ａｌ．（２００３），Ｉｎｖｅｓｔ．Ｏｐｈｔｈａｌｍｏｌ．&Ｖｉｓｕａｌ　Ｓｃｉ．４４（６）：２７４３～９（この全体の開示はこの参照により本明細書に組み込まれる）に記載された手順によって誘導する。簡潔に言うと、Ｃ５７Ｂｌ／６マウスを麻酔し、その瞳孔をトロピカミドで散大する。このマウスの網膜を、各網膜ＯＵの９、１２、及び３時の位置における１レーザース

ポットを用いてレーザー光凝固化する。レーザー光凝固化に続いて即座に、ＩＣＡＭ－１を標的としたｓｉＲＮＡを、ＯＤへ硝子体内に注射する。非特異的ｓｉＲＮＡは、対照としてＯＳへ硝子体内に注射する。

【0145】

レーザー光凝固化の後１４日で、マウスを屠殺し、Ｓａｋｕｒａｉ　Ｅ　ｅｔ　ａｌ．（２００３），ｓｕｐｒａ．に記載されたように、ＣＮＶ領域定量化のために網膜フラットマウントを準備する。簡潔に言うと、マウスに麻酔をし、胸部を開き、下行性大動脈を遮断（ｃｒｏｓｓ－ｃｌａｍｐｅｄ）する。次に右心房を挟み、フルオレセイン標識デキストランを左心室にゆっくりと注射する。

【0146】

フルオレセイン標識デキストランの注射後、眼を眼球除去し、２４時間、パラホルムアルデヒドで固定する。次に前眼房及び網膜を除去し、解析のために、各脈絡叢のフラットマウントを準備する。脈絡叢フラットマウントは、蛍光顕微鏡下で写真を撮ることによって解析し、その写真をＯｐｅｎｌａｂソフトウェアへインポートする。Ｏｐｅｎｌａｂソフトウェアを用いて、新血管新生の領域の輪郭を描き（ｏｕｔｌｉｎｅｄ）、定量化する（その際、既知のレーザー位置を蛍光タフトと比較することを必ずすること）。処理動物の新血管新生領域を、対照動物の新血管新生領域と比較する。

【図面の簡単な説明】

【0147】

【図1】図1は、未処理ＨＥＫ－２９３細胞（"未処理"）の可溶化、若しくは1、１０、５０、１００ｎｇ／ｍｌの腫瘍壊死因子アルファ（"ＴＮＦ－α"）、1、１０、１００、１０００ｎｇ／ｍｌのインターフェロンガンマ（"ＩＦＮ－γ"）、及び１００、２００、３００μＭのデスフェリオキサミン（"ＤＦＸ"）で1若しくは2日間処理したＨＥＫ－２９３細胞の可溶化液におけるヒトＩＣＡＭ－１タンパク質濃度の柱状図を示したものであり、それぞれはOD_{450}ナノメーターでＥＬＩＳＡによって測定した。

【図2】図2は、未処理ＨＥＫ－２９３細胞（"－"）の可溶化液、若しくは１００ｎｇ／ｍｌのＴＮＦ－αで処理したＨＥＫ－２９３細胞（"＋"）の可溶化液におけるヒトＩＣＡＭ－１タンパク質濃度の柱状図を示したものであり、それぞれはOD_{450}ナノメーターでＥＬＩＳＡによって測定した。前記細胞は、ｓｉＲＮＡでの形質移入なし（"なし"）、非特異的ｓｉＲＮＡで形質移入した（"ＥＧＦＰ"）、若しくはヒトＩＣＡＭ－１　ｍＲＮＡを標的とした１０個の異なるｓｉＲＮＡで形質移入した（"ｈＩＣＡＭ１＃１～１０"）ものである。

【図3】図3は、未処理ＨＥＫ－２９３細胞（"－"）の可溶化液、若しくは１００ｎｇ／ｍｌのＴＮＦ－αで処理したＨＥＫ－２９３細胞（"＋"）の可溶化液における細胞毒性の柱状図を示したものであり、それぞれはOD_{450}ナノメーターでＥＬＩＳＡによって測定した。前記細胞は、ｓｉＲＮＡでの形質移入なし（"なし"）、非特異的ｓｉＲＮＡで形質移入した（"ＥＧＦＰ"）、若しくはヒトＩＣＡＭ－１　ｍＲＮＡを標的とした１０個の異なるｓｉＲＮＡで形質移入した（"ｈＩＣＡＭ１＃１～１０"）ものである。

【図１】

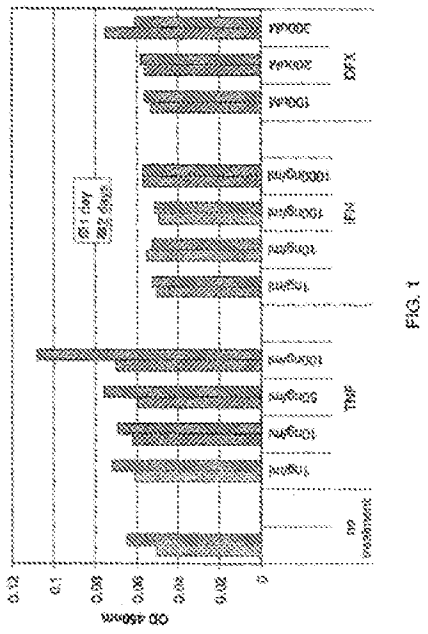

FIG. 1

【図２】

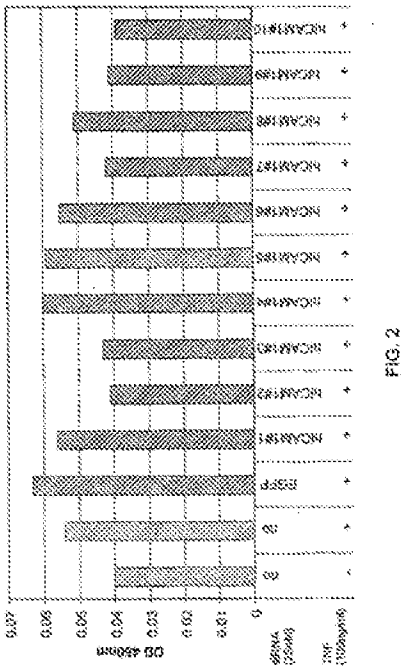

FIG. 2

【図３】

【配列表】

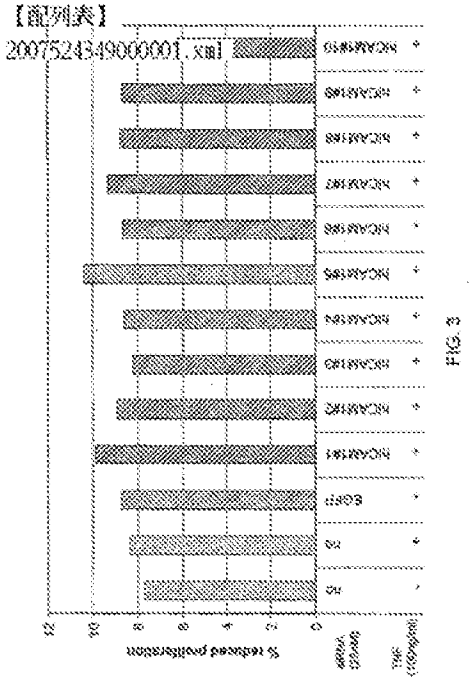

FIG. 3

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Ａ６１Ｐ　17/04	(2006.01)	Ａ６１Ｐ　17/04		
Ａ６１Ｐ　27/02	(2006.01)	Ａ６１Ｐ　27/02		
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Ａ６１Ｐ　17/06	(2006.01)	Ａ６１Ｐ　17/06		
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ZA36 ZA39 ZA40 ZA45 ZA59 ZA66 ZA81 ZA89 ZA96 ZB08
ZB11 ZB13 ZB15 ZB26 ZB27 ZB33 ZB35 ZC06 ZC35
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ZA33 ZA34 ZA36 ZA39 ZA40 ZA45 ZA59 ZA66 ZA81 ZA89
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(54) Title: RNA SEQUENCE-SPECIFIC MEDIATORS OF RNA INTERFERENCE

(57) Abstract: The present invention relates to a Drosophila in vitro system which was used to demonstrate that dsRNA is processed to RNA segments 21-23 nucleotides (nt) in length. Furthermore, when these 21-23 nt fragments are purified and added back to Drosophila extracts, they mediate RNA interference in the absence of long dsRNA. Thus , these 21-23 nt fragments are the sequence-specific mediators of RNA degradation. A molecular signal, which may be their specific length, must be present in these 21-23 nt fragments to recruit cellular factors involved in RNAi. This present invention encompasses these 21-23 nt fragments and their use for specifically inactivating gene function. The use of these fragments (or chemically synthesized oligonucleotides of the same or similar nature) enables the targeting of specific mRNAs for degradation in mammalian cells, where the use of long dsRNAs to elicit RNAi is usually not practical, presumably because of the deleterious effects of the interferon response. This specific targeting of a particular gene function is useful in functional genomic and therapeutic applications.

RNA Sequence-Specific Mediators of RNA Interference

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/265,232, filed January 31, 2001 and U.S. Provisional Application No. 60/193,594, filed March 30, 2000, and claims priority under 35 U.S.C. §119 to European Application No. 00 126 325.0 filed December 1, 2000. The entire teachings of the above applications are incorporated herein by reference.

GOVERNMENT SUPPORT

Work described herein was funded in part by grants from the National Institutes of Health through a United States Public Health Service MERIT award (Grant No. RO1-GM34277) from the National Institutes of Health. The United States government has certain rights in the invention.

BACKGROUND OF THE INVENTION

RNA interference or "RNAi" is a term initially coined by Fire and co-workers to describe the observation that double-stranded RNA (dsRNA) can block gene expression when it is introduced into worms (Fire et al. (1998) Nature 391, 806-811). dsRNA directs gene-specific, post-transcriptional silencing in many organisms, including vertebrates, and has provided a new tool for studying gene function. RNAi involves mRNA degradation, but many of the biochemical mechanisms underlying this interference are unknown. The recapitulation of the essential features of RNAi in vitro is needed for a biochemical analysis of the phenomenon.

SUMMARY OF THE INVENTION

Described herein is gene-specific, dsRNA-mediated interference in a cell-free system derived from syncytial blastoderm Drosophila embryos. The in vitro system complements genetic approaches to dissecting the molecular basis of

RNAi. As described herein, the molecular mechanisms underlying RNAi were examined using the Drosophila in vitro system. Results showed that RNAi is ATP-dependent yet uncoupled from mRNA translation. That is, protein synthesis is not required for RNAi in vitro. In the RNAi reaction, both strands (sense and antisense) of the dsRNA are processed to small RNA fragments or segments of from about 21 to about 23 nucleotides (nt) in length (RNAs with mobility in sequencing gels that correspond to markers that are 21-23 nt in length, optionally referred to as 21-23 nt RNA). Processing of the dsRNA to the small RNA fragments does not require the targeted mRNA, which demonstrates that the small RNA species is generated by processing of the dsRNA and not as a product of dsRNA-targeted mRNA degradation. The mRNA is cleaved only within the region of identity with the dsRNA. Cleavage occurs at sites 21-23 nucleotides apart, the same interval observed for the dsRNA itself, suggesting that the 21-23 nucleotide fragments from the dsRNA are guiding mRNA cleavage. That purified 21-23 nt RNAs mediate RNAi confirms that these fragments are guiding mRNA cleavage.

Accordingly, the present invention relates to isolated RNA molecules (double- stranded; single-stranded) of from about 21 to about 23 nucleotides which mediate RNAi. That is, the isolated RNAs of the present invention mediate degradation of mRNA of a gene to which the mRNA corresponds (mediate degradation of mRNA that is the transcriptional product of the gene, which is also referred to as a target gene). For convenience, such mRNA is also referred to herein as mRNA to be degraded. As used herein, the terms RNA, RNA molecule(s), RNA segment(s) and RNA fragment(s) are used interchangeably to refer to RNA that mediates RNA interference. These terms include double-stranded RNA, single-stranded RNA, isolated RNA (partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA), as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the 21-23 nt RNA or internally (at one or more nucleotides of the RNA). Nucleotides in the RNA molecules of the present invention can also comprise non-standard nucleotides, including

non-naturally occurring nucleotides or deoxyribonucleotides. Collectively, all such altered RNAs are referred to as analogs or analogs of naturally-occurring RNA. RNA of 21-23 nucleotides of the present invention need only be sufficiently similar to natural RNA that it has the ability to mediate (mediates) RNAi. As used herein the phrase "mediates RNAi" refers to (indicates) the ability to distinguish which RNAs are to be degraded by the RNAi machinery or process. RNA that mediates RNAi interacts with the RNAi machinery such that it directs the machinery to degrade particular mRNAs. In one embodiment, the present invention relates to RNA molecules of about 21 to about 23 nucleotides that direct cleavage of specific mRNA to which their sequence corresponds. It is not necessary that there be perfect correspondence of the sequences, but the correspondence must be sufficient to enable the RNA to direct RNAi cleavage of the target mRNA. In a particular embodiment, the 21-23 nt RNA molecules of the present invention comprise a 3' hydroxyl group.

The present invention also relates to methods of producing RNA molecules of about 21 to about 23 nucleotides with the ability to mediate RNAi cleavage. In one embodiment, the Drosophila in vitro system is used. In this embodiment, dsRNA is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the dsRNA is processed to RNA molecules of about 21 to about 23 nucleotides. In another embodiment, the Drosophila in vitro system is used to obtain RNA sequences of about 21 to about 23 nucleotides which mediate RNA interference of the mRNA of a particular gene (e.g., oncogene, viral gene). In this embodiment, double-stranded RNA that corresponds to a sequence of the gene to be targeted is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNA of about 21 to about 23 nucleotides in length. As shown herein, 21- 23 nt RNA mediates RNAi of the mRNA of the targeted gene (the gene whose mRNA is to be degraded). The method of obtaining 21-23 nt RNAs using the Drosophila in vitro system can further comprise isolating the RNA sequence from the combination.

The present invention also relates to 21-23 nt RNA produced by the methods of the present invention, as well as to 21-23 nt RNAs, produced by other methods, such as chemical synthesis or recombinant DNA techniques, that have the same or substantially the same sequences as naturally-occurring RNAs that mediate RNAi, such as those produced by the methods of the present invention. All of these are referred to as 21-23 nt RNAs that mediate RNA interference. As used herein, the term isolated RNA includes RNA obtained by any means, including processing or cleavage of dsRNA as described herein; production by chemical synthetic methods; and production by recombinant DNA techniques. The invention further relates to uses of the 21-23 nt RNAs, such as for therapeutic or prophylactic treatment and compositions comprising 21-23 nt RNAs that mediate RNAi, such as pharmaceutical compositions comprising 21-23 nt RNAs and an appropriate carrier (e.g., a buffer or water).

The present invention also relates to a method of mediating RNA interference of mRNA of a gene in a cell or organism (e.g., mammal such as a mouse or a human). In one embodiment, RNA of about 21 to about 23 nt which targets the mRNA to be degraded is introduced into the cell or organism. The cell or organism is maintained under conditions under which degradation of the mRNA occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism. The cell or organism can be one in which RNAi occurs as the cell or organism is obtained or a cell or organism can be one that has been modified so that RNAi occurs (e.g., by addition of components obtained from a cell or cell extract that mediate RNAi or activation of endogenous components). As used herein, the term "cell or organism in which RNAi occurs" includes both a cell or organism in which RNAi occurs as the cell or organism is obtained, or a cell or organism that has been modified so that RNAi occurs. In another embodiment, the method of mediating RNA interference of a gene in a cell comprises combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNAs of about 21 to about 23 nucleotides. 21 to 23 nt RNA is then

isolated and introduced into the cell or organism. The cell or organism is maintained under conditions in which degradation of mRNA of the gene occurs, thereby mediating RNA interference of the gene in the cell or organism. As described for the previous embodiment, the cell or organism is one in which RNAi occurs naturally (in the cell or organism as obtained) or has been modified in such a manner that RNAi occurs. 21 to 23 nt RNAs can also be produced by other methods, such as chemical synthetic methods or recombinant DNA techniques.

The present invention also relates to biochemical components of a cell, such as a Drosophila cell, that process dsRNA to RNA of about 21 to about 23 nucleotides. In addition, biochemical components of a cell that are involved in targeting of mRNA by RNA of about 21 to about 23 nucleotides are the subject of the present invention. In both embodiments, the biochemical components can be obtained from a cell in which they occur or can be produced by other methods, such as chemical synthesis or recombinant DNA methods. As used herein, the term "isolated" includes materials (e.g., biochemical components, RNA) obtained from a source in which they occur and materials produced by methods such as chemical synthesis or recombinant nucleic acid (DNA, RNA) methods.

The present invention also relates to a method for knocking down (partially or completely) the targeted gene, thus providing an alternative to presently available methods of knocking down (or out) a gene or genes. This method of knocking down gene expression can be used therapeutically or for research (e.g., to generate models of disease states, to examine the function of a gene, to assess whether an agent acts on a gene, to validate targets for drug discovery). In those instances in which gene function is eliminated, the resulting cell or organism can also be referred to as a knockout. One embodiment of the method of producing knockdown cells and organisms comprises introducing into a cell or organism in which a gene (referred to as a targeted gene) is to be knocked down, RNA of about 21 to about 23 nt that targets the gene and maintaining the resulting cell or organism under conditions under which RNAi occurs, resulting in degradation of the mRNA of the targeted gene, thereby producing knockdown cells or organisms. Knockdown cells and organisms produced by the present method are also the subject of this invention.

The present invention also relates to a method of examining or assessing the function of a gene in a cell or organism. In one embodiment, RNA of about 21 to about 23 nt which targets mRNA of the gene for degradation is introduced into a cell or organism in which RNAi occurs. The cell or organism is referred to as a test cell or organism. The test cell or organism is maintained under conditions under which degradation of mRNA of the gene occurs. The phenotype of the test cell or organism is then observed and compared to that of an appropriate control cell or organism, such as a corresponding cell or organism that is treated in the same manner except that the targeted (specific) gene is not targeted. A 21 to 23 nt RNA that does not target the mRNA for degradation can be introduced into the control cell or organism in place of the RNA introduced into the test cell or organism, although it is not necessary to do so. A difference between the phenotypes of the test and control cells or organisms provides information about the function of the degraded mRNA. In another embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract that mediates RNAi, such as the soluble extract derived from Drosophila embryo described herein, under conditions in which the double-stranded RNA is processed to generate RNA of about 21 to about 23 nucleotides. The RNA of about 21 to about 23 nucleotides is isolated and then introduced into a cell or organism in which RNAi occurs (test cell or test organism). The test cell or test organism is maintained under conditions under which degradation of the mRNA occurs. The phenotype of the test cell or organism is then observed and compared to that of an appropriate control, such as a corresponding cell or organism that is treated in the same manner as the test cell or organism except that the targeted gene is not targeted. A difference between the phenotypes of the test and control cells or organisms provides information about the function of the targeted gene. The information provided may be sufficient to identify (define) the function of the gene or may be used in conjunction with information obtained from other assays or analyses to do so.

Also the subject of the present invention is a method of validating whether an agent acts on a gene. In this method, RNA of from about 21 to about 23 nucleotides that targets the mRNA to be degraded is introduced into a cell or

organism in which RNAi occurs. The cell or organism (which contains the introduced RNA) is maintained under conditions under which degradation of mRNA occurs, and the agent is introduced into the cell or organism. Whether the agent has an effect on the cell or organism is determined; if the agent has no effect on the cell or organism, then the agent acts on the gene.

The present invention also relates to a method of validating whether a gene product is a target for drug discovery or development. RNA of from about 21 to about 23 nucleotides that targets the mRNA that corresponds to the gene for degradation is introduced into a cell or organism. The cell or organism is maintained under conditions in which degradation of the mRNA occurs, resulting in decreased expression of the gene. Whether decreased expression of the gene has an effect on the cell or organism is determined, wherein if decreased expression of the gene has an effect, then the gene product is a target for drug discovery or development.

The present invention also encompasses a method of treating a disease or condition associated with the presence of a protein in an individual comprising administering to the individual RNA of from about 21 to about 23 nucleotides which targets the mRNA of the protein (the mRNA that encodes the protein) for degradation. As a result, the protein is not produced or is not produced to the extent it would be in the absence of the treatment.

Also encompassed by the present invention is a gene identified by the sequencing of endogenous 21 to 23 nucleotide RNA molecules that mediate RNA interference.

Also encompassed by the present invention is a method of identifying target sites within an mRNA that are particularly suitable for RNAi as well as a method of assessing the ability of 21-23 nt RNAs to mediate RNAi.

BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

Figure 1 is a schematic representation of reporter mRNAs and dsRNAs Rr-Luc and Pp-Luc. Lengths and positions of the ssRNA, asRNA, and dsRNAs are shown as black bars relative to the Rr-Luc and Pp-Luc reporter mRNA sequences. Black rectangles indicate the two unrelated luciferase coding sequences, lines correspond to the 5' and 3' untranslated regions of the mRNAs.

Figure 2A is a graph of the ratio of luciferase activities after targeting 50 pM Pp- Luc mRNA with 10 nM ssRNA, asRNA, or dsRNA from the 505 bp segment of the Pp- Luc gene showing gene-specific interference by dsRNA in vitro. The data are the average values of seven trials ± standard deviation. Four independently prepared lysates were used. Luciferase activity was normalized to the buffer control; a ratio equal to one indicates no gene-specific interference.

Figure 2B is a graph of the ratio of luciferase activities after targeting 50 pM Rr- Luc mRNA with 10 nM ssRNA, asRNA, or dsRNA from the 501 bp segment of the Rr- Luc gene showing gene-specific interference by dsRNA in vitro. The data are the average values of six trials ± standard deviation. A Rr-Luc/Pp-Luc ratio equal to one indicates no gene-specific interference.

Figure 3A is a schematic representation of the experimental strategy used to show that incubation in the Drosophila embryo lysate potentiates dsRNA for gene-specific interference. The same dsRNAs used in Figure 2 (or buffer) was serially preincubated using two-fold dilutions in six successive reactions with Drosophila embryo lysate, then tested for its capacity to block mRNA expression. As a control, the same amount of dsRNA (10 nM) or buffer was diluted directly in buffer and incubated with Pp-Luc and Rr-Luc mRNAs and lysate.

Figure 3B is a graph of potentiation when targeting Pp-Luc mRNA. Black columns indicate the dsRNA or the buffer was serially preincubated; white columns correspond to a direct 32-fold dilution of the dsRNA. Values were normalized to those of the buffer controls.

Figure 3C is a graph of potentiation when targeting Rr-Luc mRNA. The corresponding buffer control is shown in Figure 3B.

Figure 4 is a graph showing effect of competitor dsRNA on gene-specific interference. Increasing concentrations of nanos dsRNA (508 bp) were added to

reactions containing 5 nM dsRNA (the same dsRNAs used in Figures 2A and 2B) targeting Pp-Luc mRNA (black columns, left axis) or Rr-Luc mRNA (white columns, right axis). Each reaction contained both a target mRNA (Pp-Luc for the black columns, Rr-Luc for the white) and an unrelated control mRNA (Rr-Luc for the black columns, Pp-Luc for the white). Values were normalized to the buffer control (not shown). The reactions were incubated under standard conditions (see Methods).

Figure 5A is a graph showing the effect of dsRNA on mRNA stability. Circles, Pp-Luc mRNA; squares, Rr-Luc mRNA; filled symbols, buffer incubation; open symbols, incubation with Pp-dsRNA.

Figure 5B is a graph showing the stability of Rr-Luc mRNA incubated with Rr- dsRNA or Pp-dsRNA. Filled squares, buffer; open squares, Pp-dsRNA (10 nM); open circles, Rr-dsRNA (10 nM).

Figure 5C is a graph showing the dependence on dsRNA length. The stability of the Pp-Luc mRNA was assessed after incubation in lysate in the presence of buffer or dsRNAs of different lengths. Filled squares, buffer; open circles, 49 bp dsRNA (10 nM); open inverted triangles, 149 bp dsRNA (10 nM); open triangles, 505 bp dsRNA (10 nM); open diamonds, 997 bp dsRNA (10 nM). Reactions were incubated under standard conditions (see Methods).

Figure 6 is a graph showing that RNAi Requires ATP. Creatine kinase (CK) uses creatine phosphate (CP) to regenerate ATP. Circles, +ATP, +CP, +CK; squares, -ATP, +CP, +CK; triangles, -ATP, -CP, +CK; inverted triangles, -ATP, +CP, -CK.

Figure 7A is a graph of protein synthesis, as reflected by luciferase activity produced after incubation of Rr-luc mRNA in the in vitro RNAi reaction for 1 hour, in the presence of the protein synthesis inhibitors anisomycin, cycloheximide, or chloramphenicol, relative to a reaction without any inhibitor showing that RNAi does not require mRNA translation.

Figure 7B is a graph showing translation of 7-methyl-guanosine- and adenosine- capped Pp-luc mRNAs (circles and squares, respectively) in the RNAi

reaction in the absence of dsRNA, as measured by luciferase activity produced in a one-hour incubation.

Figure 7C is a graph showing incubation in an RNAi reaction of uniformly ^{32}P- radiolabeled 7-methyl-guanosine-capped Pp-luc mRNA (circles) and adenosine-capped Pp-luc mRNA (squares), in the presence (open symbols) and absence (filled symbols) of 505 bp Pp-luc dsRNA.

Figure 8A is a graph of the of the denaturing agarose-gel analysis of Pp-luc mRNA incubated in a standard RNAi reaction with buffer, 505 nt Pp-asRNA, or 505 bp Pp-dsRNA for the times indicated showing that asRNA causes a small amount of RNAi in vitro.

Figure 8B is a graph of the of the denaturing agarose-gel analysis of Rr-luc mRNA incubated in a standard RNAi reaction with buffer, 505 nt Pp-asRNA, or 505 bp Pp-dsRNA for the times indicated showing that asRNA causes a small amount of RNAi in vitro.

Figure 9 is a schematic of the positions of the three dsRNAs, 'A,' 'B,' and 'C,' relative to the Rr-luc mRNA.

Figure10 indicates the cleavage sites mapped onto the first 267 nt of the Rr-luc mRNA (SEQ ID NO: 1). The blue bar below the sequence indicates the position of dsRNA 'C,' and blue circles indicate the position of cleavage sites caused by this dsRNA. The green bar denotes the position of dsRNA 'B,' and green circles, the cleavage sites. The magenta bar indicates the position of dsRNA 'A,' and magenta circles, the cleavages. An exceptional cleavage within a run of 7 uracils is marked with a red arrowhead.

Figure 11 is a proposed model for RNAi. RNAi is envisioned to begin with cleavage of the dsRNA to 21-23 nt products by a dsRNA-specific nuclease, perhaps in a multiprotein complex. These short dsRNAs might then be dissociated by an ATP- dependent helicase, possibly a component of the initial complex, to 21-23 nt asRNAs that could then target the mRNA for cleavage. The short asRNAs are imagined to remain associated with the RNAi-specific proteins (circles) that were originally bound by the full-length dsRNA, thus explaining the inefficiency of

asRNA to trigger RNAi in vivo and in vitro. Finally, a nuclease (triangles) would cleave the mRNA.

Figure 12 is a bar graph showing sequence-specific gene silencing by 21-23 nt fragments. Ratio of luciferase activity after targeting of Pp-Luc and Rr-Luc mRNA by 5 nM Pp-Luc or Rr-Luc dsRNA (500 bp) or 21-23 nt fragments isolated from a previous incubation of the respective dsRNA in Drosophila lysate. The amount of isolated 21-23 mers present in the incubation reaction correspond to approximately the same amount of 21-23 mers generated during an incubation reaction with 5 nM 500 bp dsRNA. The data are average values of 3 trials and the standard deviation is given by error bars. Luciferase activity was normalized to the buffer control.

Figure 13A illustrates the purification of RNA fragments on a Superdex HR 200 10/30 gel filtration column (Pharmacia) using the method described in Example 4. dsRNA was 32P-labeled, and the radioactivity recovered in each column fraction is graphed. The fractions were also analyzed by denaturing gel electrophoresis (inset).

Figure 13B demonstrates the ability of the Rr-luciferase RNA, after incubation in the Drosophila lysate and fractionation as in Fig. 13A, to mediate sequence-specific interference with the expression of a Rr-luciferase target mRNA. One microliter of each resuspended fraction was tested in a 10 microliter in vitro RNAi reaction (see Example 1). This procedure yields a concentration of RNA in the standard in vitro RNAi reaction that is approximately equal to the concentration of that RNA species in the original reaction prior to loading on the column. Relative luminescence per second has been normalized to the average value of the two buffer controls.

Figure 13C is the specificity control for Fig 13B. It demonstrates that the fractionated RNA of Fig 13B does not efficiently mediate sequence-specific interference with the expression of a Pp-luciferase mRNA. Assays are as in Fig 13B.

Figures 14A and 14B are schematic representations of reporter constructs and siRNA duplexes. Figure 14A illustrates the firefly (*Pp*-luc) and sea pansy (*Rr*-

luc) luciferase reporter gene regions from plasmids pGL2-Control, pGL3-Control, and pRL-TK (Promega). SV40 regulatory elements, the HSV thymidine kinase promoter, and two introns (lines) are indicated. The sequence of GL3 luciferase is 95% identical to GL2, but RL is completely unrelated to both. Luciferase expression from pGL2 is approximately 10-fold lower than from pGL3 in transfected mammalian cells. The region targeted by the siRNA duplexes is indicated as black bar below the coding region of the luciferase genes. Figure 14B shows the sense (top) and antisense (bottom) sequences of the siRNA duplexes targeting GL2 (SEQ ID Nos: 10 and 11), GL3 (SEQ ID Nos: 12 and 13), and RL (SEQ ID Nos: 14 and 15) luciferase are shown. The GL2 and GL3 siRNA duplexes differ by only 3 single nucleotide substitutions (boxed in gray). As unspecific control, a duplex with the inverted GL2 sequence, invGL2 (SEQ ID Nos: 16 and 17), was synthesized. The 2 nt 3' overhang of 2'-deoxythymidine is indicated as TT; uGL2 (SEQ ID Nos: 18 and 19) is similar to GL2 siRNA but contains ribo-uridine 3' overhangs.

Figures 15A-15J are graphs showing RNA interference by siRNA duplexes. Ratios of target to control luciferase were normalized to a buffer control (bu, black bars); gray bars indicate ratios of *Photinus pyralis* (*Pp*-luc) GL2 or GL3 luciferase to *Renilla reniformis* (*Rr*-luc) RL luciferase (left axis), white bars indicate RL to GL2 or GL3 ratios (right axis). Figures 15A, 15C, 15E, 15G, and 15I show results of experiments performed with the combination of pGL2-Control and pRL-TK reporter plasmids, Figures 15B, 15D, 15F, 15H, and 15J with pGL3-Control and pRL-TK reporter plasmids. The cell line used for the interference experiment is indicated at the top of each plot. The ratios of *Pp*-luc/*Rr*-luc for the buffer control (bu) varied between 0.5 and 10 for pGL2/pRL, and between 0.03 and 1 for pGL3/pRL, respectively, before normalization and between the various cell lines tested. The plotted data were averaged from three independent experiments ± S.D.

Figures 16A-16F are graphs showing the effects of 21 nt siRNAs, 50 bp, and 500 bp dsRNAs on luciferase expression in HeLa cells. The exact length of the long dsRNAs is indicated below the bars. Figures 16A, 16C, and 16E describe experiments performed with pGL2-Control and pRL-TK reporter plasmids, Figures 16B, 16D, and 16F with pGL3-Control and pRL-TK reporter plasmids. The data

were averaged from two independent experiments ± S.D. Figures 16A, 16B, Absolute *Pp*-luc expression, plotted in arbitrary luminescence units. Figure 16C, 16D, Rr-luc expression, plotted in arbitrary luminescence units. Figures 16E, 16F, Ratios of normalized target to control luciferase. The ratios of luciferase activity for siRNA duplexes were normalized to a buffer control (bu, black bars); the luminescence ratios for 50 or 500 bp dsRNAs were normalized to the respective ratios observed for 50 and 500 bp dsRNA from humanized GFP (hG, black bars). It should be noted, that the overall differences in sequence between the 49 and 484 bp dsRNAs targeting GL2 and GL3 are not sufficient to confer specificity between GL2 and GL3 targets (43 nt uninterrupted identity in 49 bp segment, 239 nt longest uninterrupted identity in 484 bp segment) (Parrish, S., *et al., Mol. Cell, 6:*1077-1087 (2000)).

DETAILED DESCRIPTION OF THE INVENTION

Double-stranded (dsRNA) directs the sequence-specific degradation of mRNA through a process known as RNA interference (RNAi). The process is known to occur in a wide variety of organisms, including embryos of mammals and other vertebrates. Using the Drosophila in vitro system described herein, it has been demonstrated that dsRNA is processed to RNA segments 21-23 nucleotides (nt) in length, and furthermore, that when these 21-23 nt fragments are purified and added back to Drosophila extracts, they mediate RNA interference in the absence of longer dsRNA. Thus, these 21-23 nt fragments are sequence-specific mediators of RNA degradation. A molecular signal, which may be the specific length of the fragments, must be present in these 21-23 nt fragments to recruit cellular factors involved in RNAi. This present invention encompasses these 21-23 nt fragments and their use for specifically inactivating gene function. The use of these fragments (or recombinantly produced or chemically synthesized oligonucleotides of the same or similar nature) enables the targeting of specific mRNAs for degradation in mammalian cells. Use of long dsRNAs in mammalian cells to elicit RNAi is usually not practical, presumably because of the deleterious effects of the interferon response. Specific targeting of a particular gene function, which is possible with

21-23 nt fragments of the present invention, is useful in functional genomic and therapeutic applications.

In particular, the present invention relates to RNA molecules of about 21 to about 23 nucleotides that mediate RNAi. In one embodiment, the present invention relates to RNA molecules of about 21 to about 23 nucleotides that direct cleavage of specific mRNA to which they correspond. The 21-23 nt RNA molecules of the present invention can also comprise a 3' hydroxyl group. The 21-23 nt RNA molecules can be single-stranded or double stranded (as two 21-23 nt RNAs); such molecules can be blunt ended or comprise overhanging ends (*e.g.*, 5', 3'). In specific embodiments, the RNA molecule is double stranded and either blunt ended or comprises overhanging ends (as two 21-23 nt RNAs).

In one embodiment, at least one strand of the RNA molecule has a 3' overhang from about 1 to about 6 nucleotides (*e.g.*, pyrimidine nucleotides, purine nucleotides) in length. In other embodiments, the 3' overhang is from about 1 to about 5 nucleotides, from about 1 to about 3 nucleotides and from about 2 to about 4 nucleotides in length. In one embodiment the RNA molecule is double stranded, one strand has a 3' overhang and the other strand can be blunt-ended or have an overhang. In the embodiment in which the RNA molecule is double stranded and both strands comprise an overhang, the length of the overhangs may be the same or different for each strand. In a particular embodiment, the RNA of the present invention comprises 21 nucleotide strands which are paired and which have overhangs of from about 1 to about 3, particularly about 2, nucleotides on both 3' ends of the RNA. In order to further enhance the stability of the RNA of the present invention, the 3' overhangs can be stabilized against degradation. In one embodiment, the RNA is stabilized by including purine nucleotides, such as adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, *e.g.*, substitution of uridine 2 nucleotide 3' overhangs by 2'-deoxythymidine is tolerated and does not affect the efficiency of RNAi. The absence of a 2' hydroxyl significantly enhances the nuclease resistance of the overhang in tissue culture medium.

The 21-23 nt RNA molecules of the present invention can be obtained using a number of techniques known to those of skill in the art. For example, the RNA can be chemically synthesized or recombinantly produced using methods known in the art. The 21-23 nt RNAs can also be obtained using the Drosophila in vitro system described herein. Use of the Drosophila in vitro system entails combining dsRNA with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the dsRNA is processed to RNA of about 21 to about 23 nucleotides. The Drosophila in vitro system can also be used to obtain RNA of about 21 to about 23 nucleotides in length which mediates RNA interference of the mRNA of a particular gene (e.g., oncogene, viral gene). In this embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double- stranded RNA is processed to the RNA of about 21 to about 23 nucleotides. As shown herein, 21-23 nt RNA mediates RNAi of the mRNA to be degraded. The present invention also relates to the 21-23 nt RNA molecules produced by the methods described herein.

In one embodiment, the methods described herein are used to identify or obtain 21-23 nt RNA molecules that are useful as sequence-specific mediators of RNA degradation and, thus, for inhibiting mRNAs, such as human mRNAs, that encode products associated with or causative of a disease or an undesirable condition. For example, production of an oncoprotein or viral protein can be inhibited in humans in order to prevent the disease or condition from occurring, limit the extent to which it occurs or reverse it. If the sequence of the gene to be targeted in humans is known, 21-23 nt RNAs can be produced and tested for their ability to mediate RNAi in a cell, such as a human or other primate cell. Those 21-23 nt human RNA molecules shown to mediate RNAi can be tested, if desired, in an appropriate animal model to further assess their in vivo effectiveness. Additional copies of 21-23 nt RNAs shown to mediate RNAi can be produced by the methods described herein.

The method of obtaining the 21-23 nt RNA sequence using the Drosophila in vitro system can further comprise isolating the RNA sequence from the combination. The 21-23 nt RNA molecules can be isolated using a number of techniques known to those of skill in the art. For example, gel electrophoresis can be used to separate 21-23 nt RNAs from the combination, gel slices comprising the RNA sequences removed and RNAs eluted from the gel slices. Alternatively, non-denaturing methods, such as non-denaturing column chromatography, can be used to isolate the RNA produced. In addition, chromatography (e.g., size exclusion chromatography), glycerol gradient centrifugation, affinity purification with antibody can be used to isolate 21-23 nt RNAs. The RNA-protein complex isolated from the Drosophila in vitro system can also be used directly in the methods described herein (e.g., method of mediating RNAi of mRNA of a gene). Soluble extracts derived from Drosophila embryo that mediate or RNAi are encompassed by the invention. The soluble Drosophila extract can be obtained in a variety of ways. For example, the soluble extract can be obtained from syncytial blastoderm Drosophila embryos as described in Examples 1, 2, and 3. Soluble extracts can be derived from other cells in which RNAi occurs. Alternatively, soluble extracts can be obtained from a cell that does not carry out RNAi. In this instance, the factors needed to mediate RNAi can be introduced into such a cell and the soluble extract is then obtained. The components of the extract can also be chemically synthesized and/or combined using methods known in the art.

Any dsRNA can be used in the methods of the present invention, provided that it has sufficient homology to the targeted gene to mediate RNAi. The sequence of the dsRNA for use in the methods of the present invention need not be known. Alternatively, the dsRNA for use in the present invention can correspond to a known sequence, such as that of an entire gene (one or more) or portion thereof. There is no upper limit on the length of the dsRNA that can be used. For example, the dsRNA can range from about 21 base pairs (bp) of the gene to the full length of the gene or more. In one embodiment, the dsRNA used in the methods of the present invention is about 1000 bp in length. In another embodiment, the dsRNA is about 500 bp in length. In yet another embodiment, the dsRNA is about 22 bp in length.

The 21 to 23 nt RNAs described herein can be used in a variety of ways. For example, the 21 to 23 nt RNA molecules can be used to mediate RNA interference of mRNA of a gene in a cell or organism. In a specific embodiment, the 21 to 23 nt RNA is introduced into human cells or a human in order to mediate RNA interference in the cells or in cells in the individual, such as to prevent or treat a disease or undesirable condition. In this method, a gene (or genes) that cause or contribute to the disease or undesirable condition is targeted and the corresponding mRNA (the transcriptional product of the targeted gene) is degraded by RNAi. In this embodiment, an RNA of about 21 to about 23 nucleotides that targets the corresponding mRNA (the mRNA of the targeted gene) for degradation is introduced into the cell or organism. The cell or organism is maintained under conditions under which degradation of the corresponding mRNA occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism. In a particular embodiment, the method of mediating RNA interference of a gene in a cell comprises combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNA of about 21 to about 23 nucleotides. The 21 to 23 nt RNA is then isolated and introduced into the cell or organism. The cell or organism is maintained under conditions in which degradation of mRNA of the gene occurs, thereby mediating RNA interference of the gene in the cell or organism. In the event that the 21-23nt RNA is introduced into a cell in which RNAi, does not normally occur, the factors needed to mediate RNAi are introduced into such a cell or the expression of the needed factors is induced in such a cell. Alternatively, 21 to 23 nt RNA produced by other methods (e.g., chemical synthesis, recombinant DNA production) to have a composition the same as or sufficiently similar to a 21 to 23 nt RNA known to mediate RNAi can be similarly used to mediate RNAi. Such 21 to 23 nt RNAs can be altered by addition, deletion, substitution or modification of one or more nucleotides and/or can comprise non-nucleotide materials. A further embodiment of this invention is an ex vivo method of treating cells from an individual to degrade a gene(s) that causes or is associated with a disease or

undesirable condition, such as leukemia or AIDS. In this embodiment, cells to be treated are obtained from the individual using known methods (e.g., phlebotomy or collection of bone marrow) and 21-23 nt RNAs that mediate degradation of the corresponding mRNA(s) are introduced into the cells, which are then re-introduced into the individual. If necessary, biochemical components needed for RNAi to occur can also be introduced into the cells.

The mRNA of any gene can be targeted for degradation using the methods of mediating interference of mRNA described herein. For example, any cellular or viral mRNA, can be targeted, and, as a result, the encoded protein (e.g., an oncoprotein, a viral protein), expression will be diminished. In addition, the mRNA of any protein associated with/causative of a disease or undesirable condition can be targeted for degradation using the methods described herein.

The present invention also relates to a method of examining the function of a gene in a cell or organism. In one embodiment, an RNA sequence of about 21 to about 23 nucleotides that targets mRNA of the gene for degradation is introduced into the cell or organism. The cell or organism is maintained under conditions under which degradation of mRNA of the gene occurs. The phenotype of the cell or organism is then observed and compared to an appropriate control, thereby providing information about the function of the gene. In another embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract derived from Drosophila embryo under conditions in which the double-stranded RNA is processed to generate RNA of about 21 to about 23 nucleotides. The RNA of about 21 to about 23 nucleotides is isolated and then introduced into the cell or organism. The cell or organism is maintained under conditions in which degradation of the mRNA of the gene occurs. The phenotype of the cell or organism is then observed and compared to an appropriate control, thereby identifying the function of the gene.

A further aspect of this invention is a method of assessing the ability of 21-23 nt RNAs to mediate RNAi and, particularly, determining which 21-23 nt RNA(s) most efficiently mediate RNAi. In one embodiment of the method, dsRNA corresponding to a sequence of an mRNA to be degraded is combined with

detectably labeled (e.g., end-labeled, such as radiolabeled) mRNA and the soluble extract of this invention, thereby producing a combination. The combination is maintained under conditions under which the double-stranded RNA is processed and the mRNA is degraded. The sites of the most effective cleavage are mapped by comparing the migration of the labeled mRNA cleavage products to markers of known length. 21 mers spanning these sites are then designed and tested for their efficiency in mediating RNAi.

Alternatively, the extract of the present invention can be used to determine whether there is a particular segment or particular segments of the mRNA corresponding to a gene which are more efficiently targeted by RNAi than other regions and, thus, can be especially useful target sites. In one embodiment, dsRNA corresponding to a sequence of a gene to be degraded, labeled mRNA of the gene is combined with a soluble extract that mediates RNAi, thereby producing a combination. The resulting combination is maintained under conditions under which the dsRNA is degraded and the sites on the mRNA that are most efficiently cleaved are identified, using known methods, such as comparison to known size standards on a sequencing gel.

OVERVIEW OF EXAMPLES

Biochemical analysis of RNAi has become possible with the development of the in vitro Drosophila embryo lysate that recapitulates dsRNA-dependent silencing of gene expression described in Example 1 (Tuschl et al., Genes Dev., 13:3191-7 (1999)). In the in vitro system, dsRNA, but not sense or asRNA, targets a corresponding mRNA for degradation, yet does not affect the stability of an unrelated control mRNA. Furthermore, pre-incubation of the dsRNA in the lysate potentiates its activity for target mRNA degradation, suggesting that the dsRNA must be converted to an active form by binding proteins in the extract or by covalent modification (Tuschl et al., Genes Dev., 13:3191-7 (1999)).

The development of a cell-free system from syncytial blastoderm Drosophila embryos that recapitulates many of the features of RNAi is described herein. The interference observed in this reaction is sequence-specific, is promoted by dsRNA,

but not by single-stranded RNA, functions by specific mRNA degradation, requires a minimum length of dsRNA and is most efficient with long dsRNA. Furthermore, preincubation of dsRNA potentiates its activity. These results demonstrate that RNAi is mediated by sequence specific processes in soluble reactions.

As described in Example 2, the in vitro system was used to analyze the requirements of RNAi and to determine the fate of the dsRNA and the mRNA. RNAi in vitro requires ATP, but does not require either mRNA translation or recognition of the 7-methyl-guanosine cap of the targeted mRNA. The dsRNA, but not single-stranded RNA, is processed in vitro to a population of 21-23 nt species. Deamination of adenosines within the dsRNA does not appear to be required for formation of the 21-23 nt RNAs. As described herein, the mRNA is cleaved only in the region corresponding to the sequence of the dsRNA and that the mRNA is cleaved at 21-23 nt intervals, strongly indicating that the 21-23 nt fragments from the dsRNA are targeting the cleavage of the mRNA. Furthermore, as described in Examples 3 and 4, when the 21-23 nt fragments are purified and added back to the soluble extract, they mediate RNA.

The present invention is illustrated by the following examples, which are not intended to be limiting in any way.

Example 1 Targeted mRNA degradation by double-stranded RNA in vitro

Materials and Methods

RNAs

Rr-Luc mRNA consisted of the 926 nt Rr luciferase coding sequence flanked by 25 nt of 5' untranslated sequence from the pSP64 plasmid polylinker and 25 nt of 3' untranslated sequence consisting of 19 nt of pSP64 plasmid polylinker sequence followed by a 6 nt Sac I site. Pp-Luc mRNA contained the 1653 nt Pp luciferase coding sequence with a Kpn I site introduced immediately before the Pp luciferase stop codon. The Pp coding sequence was flanked by 5' untranslated sequences consisting of 21 nt of pSP64 plasmid polylinker followed by the 512 nt of the 5' untranslated region (UTR) from the Drosophila hunchback mRNA and 3' untranslated sequences consisting of the 562 nt hunchback 3' UTR followed by a 6

nt Sac I site. The hunchback 3' UTR sequences used contained six G-to-U mutations that disrupt function of the Nanos Response Elements in vivo and in vitro. Both reporter mRNAs terminated in a 25 nt poly(A) tail encoded in the transcribed plasmid. For both Rr-Luc and Pp -Luc mRNAs, the transcripts were generated by run-off transcription from plasmid templates cleaved at an Nsi I site that immediately followed the 25 nt encoded poly(A) tail. To ensure that the transcripts ended with a poly(A) tail, the Nsi I-cleaved transcription templates were resected with T4 DNA Polymerase in the presence of dNTPs. The SP6 mMessage mMachine kit (Ambion) was used for in vitro transcription. Using this kit, about 80% of the resulting transcripts are 7-methyl guanosine capped. ^{32}P-radiolabeling was accomplished by including α-^{32}P-UTP in the transcription reaction.

For Pp -Luc, ss, as, and dsRNA corresponded to positions 93 to 597 relative to the start of translation, yielding a 505 bp dsRNA. For Rr -Luc, ss, as, and dsRNA corresponded to positions 118 to 618 relative to the start of translation, yielding a 501 bp dsRNA. The Drosophila nanos competitor dsRNA corresponded to positions 122 to 629 relative to the start of translation, yielding a 508 bp dsRNA. ssRNA, asRNA, and dsRNA (diagrammed in Figure 1) were transcribed in vitro with T7 RNA polymerase from templates generated by the polymerase chain reaction. After gel purification of the T7 RNA transcripts, residual DNA template was removed by treatment with RQ1 DNase (Promega). The RNA was then extracted with phenol and chloroform, and then precipitated and dissolved in water.

RNA annealing and native gel electrophoresis.

ssRNA and asRNA (0.5 μM) in 10 mM Tris-HCl (pH 7.5) with 20 mM NaCl were heated to 95 ° C for 1 min then cooled and annealed at room temperature for 12 to 16 h. The RNAs were precipitated and resuspended in lysis buffer (below). To monitor annealing, RNAs were electrophoresed in a 2% agarose gel in TBE buffer and stained with ethidium bromide (Sambrook et al., Molecular Cloning. Cold Spring Harbor Laboratory Press, Plainview, NY. (1989)).

Lysate preparation

Zero- to two-hour old embryos from Oregon R flies were collected on yeasted molasses agar at 25°C. Embryos were dechorionated for 4 to 5 min in 50% (v/v) bleach, washed with water, blotted dry, and transferred to a chilled Potter-Elvehjem tissue grinder (Kontes). Embryos were lysed at 4°C in one ml of lysis buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) containing 5 mM dithiothreitol (DTT) and 1 mg/ml Pefabloc SC (Boehringer-Mannheim) per gram of damp embryos. The lysate was centrifuged for 25 min at 14,500 x g at 4°C, and the supernatant flash frozen in aliquots in liquid nitrogen and stored at -80°C.

Reaction conditions

Lysate preparation and reaction conditions were derived from those described by Hussain and Leibowitz (Hussain and Leibowitz, Gene 46:13-23 (1986)). Reactions contained 50% (v/v) lysate, mRNAs (10 to 50 pM final concentration), and 10% (v/v) lysis buffer containing the ssRNA, asRNA, or dsRNA (10 nM final concentration). Each reaction also contained 10 mM creatine phosphate, 10 μg/ml creatine phosphokinase, 100 μM GTP, 100 μM UTP, 100 μM CTP, 500 μM ATP, 5 μM DTT, 0.1 U/mL RNasin (Promega), and 100 μM of each amino acid. The final concentration of potassium acetate was adjusted to 100 mM. For standard conditions, the reactions were assembled on ice and then pre-incubated at 25° C for 10 min before adding mRNA. After adding mRNAs, the incubation was continued for an additional 60 min. The 10 min preincubation step was omitted for the experiments in Figures 3A-3C and 5A-5C. Reactions were quenched with four volumes of 1.25x Passive Lysis Buffer (Promega). Pp and Rr luciferase activity was detected in a Monolight 2010 Luminometer (Analytical Luminescence Laboratory) using the Dual-Luciferase Reporter Assay System (Promega).

RNA stability

Reactions with ^{32}P-radiolabeled mRNA were quenched by the addition of 40 volumes of 2x PK buffer (200 mM Tris-HCl, pH 7.5, 25 mM EDTA, 300 mM NaCl,

2% w/v sodium dodecyl sulfate). Proteinase K (E.M. Merck; dissolved in water) was added to a final concentration of 465 μg/ml. The reactions were then incubated for 15 min at 65° C, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with an equal volume of isopropanol. Reactions were analyzed by electrophoresis in a formaldehyde/agarose (0.8% w/v) gel (Sambrook et al., Molecular Cloning. Cold Spring Harbor Laboratory Press, Plainview, NY. (1989)). Radioactivity was detected by exposing the agarose gel [dried under vacuum onto Nytran Plus membrane (Amersham)] to an image plate (Fujix) and quantified using a Fujix Bas 2000 and Image Gauge 3.0 (Fujix) software.

Commercial lysates

Untreated rabbit reticulocyte lysate (Ambion) and wheat germ extract (Ambion) reactions were assembled according to the manufacturer's directions. dsRNA was incubated in the lysate at 27°C (wheat germ) or 30°C (reticulocyte lysate) for 10 min prior to the addition of mRNAs.

Results and Discussion

To evaluate if dsRNA could specifically block gene expression in vitro, reporter mRNAs derived from two different luciferase genes that are unrelated both in sequence and in luciferin substrate specificity were used: Renilla reniformis (sea pansy) luciferase (Rr-Luc) and Photuris pennsylvanica (firefly) luciferase (Pp-Luc). dsRNA generated from one gene was used to target that luciferase mRNA whereas the other luciferase mRNA was an internal control co-translated in the same reaction. dsRNAs of approximately 500 bp were prepared by transcription of polymerase-chain reaction products from the Rr-Luc and Pp-Luc genes. Each dsRNA began ~100 bp downstream of the start of translation (Figure 1). Sense (ss) and anti-sense (as) RNA were transcribed in vitro and annealed to each other to produce the dsRNA. Native gel electrophoresis of the individual Rr 501 and Pp 505 nt as RNA and ssRNA used to form the Rr and Pp dsRNAs was preformed. The ssRNA, asRNA, and dsRNAs were each tested for their ability to block specifically

expression of their cognate mRNA but not the expression of the unrelated internal control mRNA.

The ssRNA, asRNA, or dsRNA was incubated for 10 min in a reaction containing Drosophila embryo lysate, then both Pp-Luc and Rr-Luc mRNAs were added and the incubation continued for an additional 60 min. The Drosophila embryo lysate efficiently translates exogenously transcribed mRNA under the conditions used. The amounts of Pp-Luc and Rr-Luc enzyme activities were measured and were used to calculate ratios of either Pp-Luc/Rr-Luc (Figure 2A) or Rr-Luc/Pp-Luc (Figure 2B). To facilitate comparison of different experiments, the ratios from each experiment were normalized to the ratio observed for a control in which buffer was added to the reaction in place of ssRNA, asRNA, or dsRNA.

Figure 2A shows that a 10 nM concentration of the 505 bp dsRNA identical to a portion of the sequence of the Pp-Luc gene specifically inhibited expression of the Pp- Luc mRNA but did not affect expression of the Rr-Luc internal control. Neither ssRNA nor asRNA affected expression of Pp-Luc or the Rr-Luc internal control. Thus, Pp-Luc expression was specifically inhibited by its cognate dsRNA. Conversely, a 10 nM concentration of the 501 bp dsRNA directed against the Rr-Luc mRNA specifically inhibited Rr-Luc expression but not that of the Pp-Luc internal control (Figure 2B). Again, comparable levels of ssRNA or asRNA had little or no effect on expression of either reporter mRNA. On average, dsRNA reduced specific luciferase expression by 70% in these experiments, in which luciferase activity was measured after 1 h incubation. In other experiments in which the translational capacity of the reaction was replenished by the addition of fresh lysate and reaction components, a further reduction in targeted luciferase activity relative to the internal control was observed.

The ability of dsRNA but not asRNA to inhibit gene expression in these lysates is not merely a consequence of the greater stability of the dsRNA (half-life about 2 h) relative to the single-stranded RNAs (half-life ~ 10 min). ssRNA and asRNA transcribed with a 7-methyl guanosine cap were as stable in the lysate as uncapped dsRNA, but do not inhibit gene expression. In contrast, dsRNA formed

from the capped ssRNA and asRNA specifically blocks expression of the targeted mRNA.

Effective RNAi in Drosophila requires the injection of about 0.2 fmol of dsRNA into a syncytial blastoderm embryo (Kennerdell and Carthew, Cell 95:1017-1026 (1998); Carthew, www1.pitt.edu/~carthew/manual/RNAi_Protocol.html (1999)). Since the average volume of a Drosophila embryo is approximately 7.3 nl, this corresponds to an intracellular concentration of about 25 nM (Mazur et al., Cryobiology 25:543-544 (1988)). Gene expression in the Drosophila lysate was inhibited by a comparable concentration of dsRNA (10 nM), but lowering the dsRNA concentration ten-fold decreased the amount of specific interference. Ten nanomolar dsRNA corresponds to a 200-fold excess of dsRNA over target mRNA added to the lysate. To test if this excess of dsRNA might reflect a time- and/or concentration-dependent step in which the input dsRNA was converted to a form active for gene-specific interference, the effect of preincubation of the dsRNA on its ability to inhibit expression of its cognate mRNA was examined. Because the translational capacity of the lysates is significantly reduced after 30 min of incubation at 25°C (unpublished observations), it was desired to ensure that all factors necessary for RNAi remained active throughout the pre-incubation period. Therefore, every 30 min, a reaction containing dsRNA and lysate was mixed with a fresh reaction containing unincubated lysate (Figure 3A). After six successive serial transfers spanning 3 hours of preincubation, the dsRNA, now diluted 64-fold relative to its original concentration, was incubated with lysate and 50 pM of target mRNA for 60 min. Finally, the Pp-Luc and Rr-Luc enzyme levels were measured. For comparison, the input amount of dsRNA (10 nM) was diluted 32-fold in buffer, and its capacity to generate gene-specific dsRNA interference in the absence of any preincubation step was assessed.

The preincubation of the dsRNA in lysate significantly potentiated its capacity to inhibit specific gene expression. Whereas the dsRNA diluted 32-fold showed no effect, the preincubated dsRNA was, within experimental error, as potent as undiluted dsRNA, despite having undergone a 64-fold dilution. Potentiation of

the dsRNA by preincubation was observed for dsRNAs targeting both the Pp-Luc mRNA (Figure 3B) and the Rr-Luc mRNA (Figure 3C). Taking into account the 64-fold dilution, the activation conferred by preincubation allowed a 156 pM concentration of dsRNA to inhibit 50 pM target mRNA. Further, dilution of the "activated" dsRNA may be effective but has not been tested. We note that although both dsRNAs tested were activated by the preincubation procedure, each fully retained its specificity to interfere with expression only of the mRNA to which it is homologous. Further study of the reactions may provide a route to identifying the mechanism of dsRNA potentiation.

One possible explanation for the observation that preincubation of the dsRNA enhances its capacity to inhibit gene expression in these lysates is that specific factors either modify and/or associate with the dsRNA. Accordingly, the addition of increasing amounts of dsRNA to the reaction might titrate such factors and decrease the amount of gene-specific interference caused by a second dsRNA of unrelated sequence. For both Pp-Luc mRNA and Rr-Luc mRNA, addition of increasing concentrations of the unrelated Drosophila nanos dsRNA to the reaction decreased the amount of gene- specific interference caused by dsRNA targeting the reporter mRNA (Figure 4). None of the tested concentrations of nanos dsRNA affected the levels of translation of the untargeted mRNA, demonstrating that the nanos dsRNA specifically titrated factors involved in gene-specific interference and not components of the translational machinery. The limiting factor(s) was titrated by addition of approximately 1000 nM dsRNA, a 200-fold excess over the 5 nM of dsRNA used to produce specific interference.

Interference in vitro might reflect either a specific inhibition of mRNA translation or the targeted destruction of the specific mRNA. To distinguish these two possibilities, the fates of the Pp-Luc and Rr-Luc mRNAs were examined directly using ^{32}P-radiolabeled substrates. Stability of 10 nM Pp-Luc mRNA or Rr-Luc mRNA incubated in lysate with either buffer or 505 bp Pp-dsRNA (10 nM). Samples were deproteinized after the indicated times and the ^{32}P-radiolabeled mRNAs were then resolved by denaturing gel electrophoresis. In the absence of dsRNA, both the Pp-Luc and Rr-Luc mRNAs were stable in the lysates, with ~ 75%

of the input mRNA remaining after 3 h of incubation. (About 25% of the input mRNA is rapidly degraded in the reaction and likely represents uncapped mRNA generated by the in vitro transcription process.) In the presence of dsRNA (10 nM, 505 bp) targeting the Pp-Luc mRNA, less than 15% of the Pp-Luc mRNA remained after 3 h (Figure 5A). As expected, the Rr-Luc mRNA remained stable in the presence of the dsRNA targeting Pp-Luc mRNA. Conversely, dsRNA (10 nM, 501 bp) targeting the Rr-Luc mRNA caused the destruction of the Rr-Luc mRNA but had no effect on the stability of Pp-Luc mRNA (Figure 5B). Thus, the dsRNA specifically caused accelerated decay of the mRNA to which it is homologous with no effect on the stability of the unrelated control mRNA. This finding indicates that in vivo, at least in Drosophila, the effect of dsRNA is to directly destabilize the target mRNA, not to change the subcellular localization of the mRNA, for example, by causing it to be specifically retained in the nucleus, resulting in non-specific degradation.

These results are consistent with the observation that RNAi leads to reduced cytoplasmic mRNA levels in vivo, as measured by in situ hybridization (Montgomery et al., Proc. Natl. Acad. Sci. USA 95:15502-15507 (1998)) and Northern blotting (Ngo et al., Proc. Natl. Acad. Sci. USA 95:14687-14692 (1998)). Northern blot analyses in trypanosomes and hydra suggest that dsRNA typically decreases mRNA levels by less than 90% (Ngo et al., Proc. Natl. Acad. Sci. USA 95:14687-14692 (1998); Lohmann et al., Dev. Biol. 214:211-214 (1999)). The data presented here show that in vitro mRNA levels are reduced 65 to 85% after three hours incubation, an effect comparable with observations in vivo. They also agree with the finding that RNAi in C. elegans is post- transcriptional (Montgomery et al., Proc. Natl. Acad. Sci. USA 95:15502-15507 (1998)). The simplest explanation for the specific effects on protein synthesis is that it reflects the accelerated rate of RNA decay. However, the results do not exclude independent but specific effects on translation as well as stability.

In vivo, RNAi appears to require a minimum length of dsRNA (Ngo et al., Proc. Natl. Acad. Sci., USA, 95:14687-14692 (1998)). The ability of RNA duplexes of lengths 49 bp, 149 bp, 505 bp, and 997 bp (diagrammed in Figure 1) to target the

degradation of the Pp-Luc mRNA in vitro was assessed. In good agreement with in vivo observations, the 49 bp dsRNA was ineffective in vitro, while the 149 bp dsRNA enhanced mRNA decay only slightly, and both the 505 and 997 bp dsRNAs caused robust mRNA degradation (Figure 5C). 50bp dsRNA targeting other portions of the mRNA cause detectable mRNA degradation, though not as robust as that seen for 500bp dsRNA. Thus, although some short dsRNA do not mediate RNAi, others of approximately the same length, but different composition, will be able to do so.

Whether the gene-specific interference observed in Drosophila lysates was a general property of cell-free translation systems was examined. The effects of dsRNAs on expression of Pp-Luc and Rr-Luc mRNA were examined in commercially available wheat germ extracts and rabbit reticulocyte lysates. There was no effect of addition of 10 nM of either ssRNA, asRNA, or dsRNA on the expression of either mRNA reporter in wheat germ extracts. In contrast, the addition of 10 nM of dsRNA to the rabbit reticulocyte lysate caused a profound and rapid, non-specific decrease in mRNA stability. For example, addition of Rr-Luc dsRNA caused degradation of both Rr-Luc and Pp-Luc mRNAs within 15 min. The same non-specific effect was observed upon addition of Pp-Luc dsRNA. The non-specific destruction of mRNA induced by the addition of dsRNA to the rabbit reticulocyte lysate presumably reflects the previously observed activation of RNase L by dsRNA (Clemens and Williams, Cell 13:565-572 (1978); Williams et al., Nucleic Acids Res. 6:1335-1350 (1979); Zhou et al., Cell 72:753-765 (1993); Matthews, Interactions between Viruses and the Cellular Machinery for Protein Synthesis. In Translational Control (eds. J. Hershey, M. Mathews and N. Sonenberg), pp. 505-548. Cold Spring Harbor Laboratory Press, Plainview, NY. (1996)). Mouse cell lines lacking dsRNA-induced anti-viral pathways have recently been described (Zhou et al., Virology 258:435-440 (1999)) and may be useful in the search for mammalian RNAi. Although RNAi is known to exist in some mammalian cells (Wianny and Zernicka-Goetz Nat. Cell Biol. 2: 70-75 (2000)), in many mammalian cell types its presence is likely obscured by the rapid induction by dsRNA of non-specific anti-viral responses.

dsRNA-targeted destruction of specific mRNA is characteristic of RNAi, which has been observed in vivo in many organisms, including Drosophila. The system described above recapitulates in a reaction in vitro many aspects of RNAi. The targeted mRNA is specifically degraded whereas unrelated control mRNAs present in the same solution are not affected. The process is most efficient with dsRNAs greater than 150 bp in length. The dsRNA-specific degradation reaction in vitro is probably general to many, if not all, mRNAs since it was observed using two unrelated genes.

The magnitude of the effects on mRNA stability in vitro described herein are comparable with those reported in vivo (Ngo et al., Proc. Natl. Acad. Sci., USA, 95:14687-14692 (1998); Lohmann et al., Dev. Biol., 214:211-214 (1999). However, the reaction in vitro requires an excess of dsRNA relative to mRNA. In contrast, a few molecules of dsRNA per cell can inhibit gene expression in vivo (Fire et al., Nature, 391: 806-811 (1998); Kennerdell and Carthew, Cell, 95:1017-1026 (1998)). The difference between the stoichiometry of dsRNA to target mRNA in vivo and in vitro should not be surprising in that most in vitro reactions are less efficient than their corresponding in vivo processes. Interestringly, incubation of the dsRNA in the lysate greatly potentiated its activity for RNAi, indicating that it is either modified or becomes associated with other factors or both. Perhaps a small number of molecules is effective in inhibiting the targeted mRNA in vivo because the injected dsRNA has been activated by a process similar to that reported here for RNAi in Drosophila lysates.

Example 2 Double-Stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals

Methods and Material

In vitro RNAi

In vitro RNAi reactions and lysate preparation were as described in Example 1 (Tuschl et al., Genes Dev., 13:3191-7 (1999)) except that the reaction contained 0.03 g/ml creatine kinase, 25 μM creatine phosphate (Fluka), and 1 mM ATP.

Creatine phosphate was freshly dissolved at 500 mM in water for each experiment. GTP was omitted from the reactions, except in Figures 2 and 3.

RNA Synthesis.

Pp-luc and Rr-luc mRNAs and Pp- and Rr-dsRNAs (including dsRNA 'B' in Figure 6) were synthesized by in vitro transcription as described previously (Tuschl et al., Genes Dev., 13:3191-7 (1999)). To generate transcription templates for dsRNA 'C,' the 5′ sense RNA primer was gcgtaatacgactcactataGAACAAAGGAAACGGATGAT (SEQ ID NO: 2) and the 3′ sense RNA primer was GAAGAAGTTATTCTCCAAAA (SEQ ID NO: 3); the 5′ asRNA primer was gcgtaatacgactcactataGAAGAAGTTATTCTCCAAAA (SEQ ID NO: 4)and the 3′ asRNA primer was GAACAAAGGAAACGGATGAT (SEQ ID NO: 5). For dsRNA 'A' the 5′ sense RNA primer was gcgtaatacgactcactataGTAGCGCGGTGTATTATACC (SEQ ID NO: 6)and the 3′ sense RNA primer was GTACAACGTCAGGTTTACCA (SEQ ID NO: 7); the 5′ asRNA primer was gcgtaatacgactcactataGTACAACGTCAGGTTTACCA (SEQ ID NO: 8)and the 3′ asRNA primer was GTAGCGCGGTGTATTATACC (SEQ ID NO: 9) (lowercase, T7 promoter sequence).

mRNAs were 5′-end-labeled using guanylyl transferase (Gibco/BRL), S-adenosyl methionine (Sigma), and α-^{32}P-GTP (3000 Ci/mmol; New England Nuclear) according to the manufacturer's directions. Radiolabeled RNAs were purified by poly(A) selection using the Poly(A) Tract III kit (Promega). Nonradioactive 7-methyl- guanosine- and adenosine-capped RNAs were synthesized in in vitro transcription reactions with a 5-fold excess of 7-methyl-G(5′)ppp(5′)G or A(5′)ppp(5′)G relative to GTP. Cap analogs were purchased from New England Biolabs.

ATP depletion and Protein Synthesis Inhibition

ATP was depleted by incubating the lysate for 10 minutes at 25°C with 2 mM glucose and 0.1 U/ml hexokinase (Sigma). Protein synthesis inhibitors were purchased from Sigma and dissolved in absolute ethanol as 250-fold concentrated

stocks. The final concentrations of inhibitors in the reaction were: anisomycin, 53 mg/ml; cycloheximide, 100 mg/ml; chloramphenicol, 100 mg/ml. Relative protein synthesis was determined by measuring the activity of Rr luciferase protein produced by translation of the Rr-luc mRNA in the RNAi reaction after 1 hour as described previously (Tuschl et al., Genes Dev., 13:3191-7 (1999)).

Analysis of dsRNA Processing

Internally α-^{32}P-ATP-labeled dsRNAs (505 bp Pp-luc or 501 Rr-luc) or 7-methyl-guanosine-capped Rr-luc antisense RNA (501 nt) were incubated at 5 nM final concentration in the presence or absence of unlabeled mRNAs in Drosophila lysate for 2 hours in standard conditions. Reactions were stopped by the addition of 2x proteinase K buffer and deproteinized as described previously (Tuschl et al., Genes Dev., 13:3191- 3197 (1999)). Products were analyzed by electrophoresis in 15% or 18% polyacrylamide sequencing gels. Length standards were generated by complete RNase T1 digestion of α-^{32}P-ATP-labeled 501 nt Rr-luc sense RNA and asRNA.

For analysis of mRNA cleavage, 5′-^{32}P-radiolabeled mRNA (described above) was incubated with dsRNA as described previously (Tuschl et al., Genes Dev., 13:3191- 3197 (1999)) and analyzed by electrophoresis in 5% (Figure 5B) and 6% (Figure 6C) polyacrylamide sequencing gels. Length standards included commercially available RNA size standards (FMC Bioproducts) radiolabeled with guanylyl transferase as described above and partial base hydrolysis and RNase T1 ladders generated from the 5′-radiolabeled mRNA.

Deamination Assay

Internally α-^{32}P-ATP-labeled dsRNAs (5 nM) were incubated in Drosophila lysate for 2 hours at standard conditions. After deproteinization, samples were run on 12% sequencing gels to separate full-length dsRNAs from the 21-23 nt products. RNAs were eluted from the gel slices in 0.3 M NaCl overnight, ethanol-precipitated, collected by centrifugation, and redissolved in 20 µl water. The RNA was hydrolyzed into nucleoside 5 -phosphates with nuclease P1 (10 µl reaction containing 8 µl RNA in water, 30 mM KOAc pH 5.3, 10 mM $ZnSO_4$, 10 µg or 3

units nuclease P1, 3 hours, 50° C). Samples (1 ml) were co-spotted with non-radioactive 5 -mononucleotides [0.05 O.D. units (A_{260}) of pA, pC, pG, pI, and pU] on cellulose HPTLC plates (EM Merck) and separated in the first dimension in isobutyric acid/25% ammonia/water (66/1/33, v/v/v) and in the second dimension in 0.1M sodium phosphate, pH 6.8/ammonium sulfate/1-propanol (100/60/2, v/w/v; Silberklang et al., 1979). Migration of the non- radioactive internal standards was determined by UV-shadowing.

Results and Discussion

RNAi Requires ATP

As described in Example 1, Drosophila embryo lysates faithfully recapitulate RNAi (Tuschl et al., Genes Dev., 13:3191-7 (1999)). Previously, dsRNA-mediated gene silencing was monitored by measuring the synthesis of luciferase protein from the targeted mRNA. Thus, these RNAi reactions contained an ATP-regenerating system, needed for the efficient translation of the mRNA. To test if ATP was, in fact, required for RNAi, the lysates were depleted for ATP by treatment with hexokinase and glucose, which converts ATP to ADP, and RNAi was monitored directly by following the fate of ^{32}P-radiolabeled Renilla reniformis luciferase (Rr-luc) mRNA (Figure 6). Treatment with hexokinase and glucose reduced the endogenous ATP level in the lysate from 250 μM to below 10 μM. ATP regeneration required both exogenous creatine phosphate and creatine kinase, which acts to transfer a high-energy phosphate from creatine phosphate to ADP. When ATP-depleted extracts were supplemented with either creatine phosphate or creatine kinase separately, no RNAi was observed. Therefore, RNAi requires ATP in vitro. When ATP, creatine phosphate, and creatine kinase were all added together to reactions containing the ATP-depleted lysate, dsRNA-dependent degradation of the Rr-luc mRNA was restored (Figure 6). The addition of exogenous ATP was not required for efficient RNAi in the depleted lysate, provided that both creatine phosphate and creatine kinase were present, demonstrating that the endogenous concentration (250 mM) of adenosine nucleotide is sufficient to support RNAi. RNAi with a Photinus pyralis luciferase (Pp-luc) mRNA was also ATP-dependent.

The stability of the Rr-luc mRNA in the absence of Rr-dsRNA was reduced in ATP-depleted lysates relative to that observed when the energy regenerating system was included, but decay of the mRNA under these conditions did not display the rapid decay kinetics characteristic of RNAi in vitro, nor did it generate the stable mRNA cleavage products characteristic of dsRNA-directed RNAi. These experiments do not establish if the ATP requirement for RNAi is direct, implicating ATP in one or more steps in the RNAi mechanism, or indirect, reflecting a role for ATP in maintaining high concentrations of another nucleoside triphosphate in the lysate.

Translation Is Not Required for RNAi In Vitro

The requirement for ATP suggested that RNAi might be coupled to mRNA translation, a highly energy-dependent process. To test this possibility, various inhibitors of protein synthesis were added to the reaction by preparing a denaturing agarose-gel analysis of 5′-32P-radiolabeled Pp-luc mRNA after incubation for indicated times in a standard RNAi reaction with and without protein synthesis inhibitors. The eukaryotic translation inhibitors anisomycin, an inhibitor of initial peptide bond formation, cycloheximide, an inhibitor of peptide chain elongation, and puromycin, a tRNA mimic which causes premature termination of translation (Cundliffe, Antibiotic Inhibitors of Ribosome Function. In The Molecular Basis of Antibiotic Action, E. Gale, E. Cundliffe, P. Reynolds, M. Richmond and M. Warning, eds. (New York: Wiley), pp. 402-547. (1981)) were tested. Each of these inhibitors reduced protein synthesis in the Drosophila lysate by more than 1,900-fold (Figure 7A). In contrast, chloramphenicol, an inhibitor of Drosophila mitochondrial protein synthesis (Page and Orr-Weaver, Dev. Biol., 183:195-207 (1997)), had no effect on translation in the lysates (Figure 7A). Despite the presence of anisomycin, cycloheximide, or chloramphenicol, RNAi proceeded at normal efficiency. Puromycin also did not perturb efficient RNAi. Thus, protein synthesis is not required for RNAi in vitro.

Translational initiation is an ATP-dependent process that involves recognition of the 7-methyl guanosine cap of the mRNA (Kozak, Gene, 234:187-208

(1999); Merrick and Hershey, The Pathway and Mechanism of Eukaryotic Protein Synthesis. In Translational Control, J. Hershey, M. Mathews and N. Sonenberg, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 31-69 (1996)). The Drosophila lysate used to support RNAi in vitro also recapitulates the cap-dependence of translation; Pp-luc mRNA with a 7-methyl-guanosine cap was translated greater than ten-fold more efficiently than was the same mRNA with an A(5′)ppp(5′)G cap (Figure 7B). Both RNAs were equally stable in the Drosophila lysate, showing that this difference in efficiency cannot be merely explained by more rapid decay of the mRNA with an adenosine cap (see also Gebauer et al., EMBO J., 18:6146-54 (1999)). Although the translational machinery can discriminate between Pp-luc mRNAs with 7- methyl-guanosine and adenosine caps, the two mRNAs were equally susceptible to RNAi in the presence of Pp-dsRNA (Figure 7C). These results suggest that steps in cap recognition are not involved in RNAi.

dsRNA Is Processed to 21-23 nt Species

RNAs 25 nt in length are generated from both the sense and anti-sense strands of genes undergoing post-transcriptional gene silencing in plants (Hamilton and Baulcombe, Science, 286:950-2 (1999)). Denaturing acrylamide-gel analysis of the products formed in a two-hour incubation of uniformly ^{32}P-radiolabeled dsRNAs and capped asRNA in lysate under standard RNAi conditions, in the presence or absence of target mRNAs. It was found that dsRNA is also processed to small RNA fragments. When incubated in lysate, approximately 15% of the input radioactivity of both the 501 bp Rr-dsRNA and the 505 bp Pp-dsRNA appeared in 21 to 23 nt RNA fragments. Because the dsRNAs are more than 500 bp in length, the 15% yield of fragments implies that multiple 21-23 nt RNAs are produced from each full-length dsRNA molecule. No other stable products were detected. The small RNA species were produced from dsRNAs in which both strands were uniformly ^{32}P-radiolabeled. Formation of the 21-23 nt RNAs from the dsRNA did not require the presence of the corresponding mRNA, demonstrating that the small RNA species is generated by processing of the dsRNA, rather than as a product of

dsRNA-targeted mRNA degradation. It was noted that 22 nucleotides corresponds to two turns of an A-form RNA-RNA helix.

When dsRNAs radiolabeled within either the sense or the anti-sense strand were incubated with lysate in a standard RNAi reaction, 21-23 nt RNAs were generated with comparable efficiency. These data support the idea that the 21-23 nt RNAs are generated by symmetric processing of the dsRNA. A variety of data support the idea that the 21-23 nt RNA is efficiently generated only from dsRNA and is not the consequence of an interaction between single-stranded RNA and the dsRNA. First, a ^{32}P-radiolabeled 505 nt Pp-luc sense RNA or asRNA was not efficiently converted to the 21-23 nt product when it was incubated with 5 nM nonradioactive 505 bp Pp- dsRNA. Second, in the absence of mRNA, a 501 nt 7-methyl-guanosine-capped Rr- asRNA produced only a barely detectable amount of 21-23 nt RNA (capped single- stranded RNAs are as stable in the lysate as dsRNA, Tuschl et al., Genes Dev., 13:3191- 7 (1999)), probably due to a small amount of dsRNA contaminating the anti-sense preparation. However, when Rr-luc mRNA was included in the reaction with the ^{32}P- radiolabeled, capped Rr-asRNA, a small amount of 21-23 nt product was generated, corresponding to 4% of the amount of 21-23 nt RNA produced from an equimolar amount of Rr-dsRNA. This result is unlikely to reflect the presence of contaminating dsRNA in the Rr-asRNA preparation, since significantly more product was generated from the asRNA in the presence of the Rr-luc mRNA than in the absence. Instead, the data suggest that asRNA can interact with the complementary mRNA sequences to form dsRNA in the reaction and that the resulting dsRNA is subsequently processed to the small RNA species. Rr-asRNA can support a low level of bona fide RNAi in vitro (see below), consistent with this explanation.

It was next asked if production of the 21-23 nt RNAs from dsRNA required ATP. When the 505 bp Pp-dsRNA was incubated in a lysate depleted for ATP by treatment with hexokinase and glucose, 21-23 nt RNA was produced, albeit 6 times slower than when ATP was regenerated in the depleted lysate by the inclusion of creatine kinase and creatine phosphate. Therefore, ATP may not be required for production of the 21-23 nt RNA species, but may instead simply enhance its

formation. Alternatively, ATP may be required for processing of the dsRNA, but at a concentration less than that remaining after hexokinase treatment. The molecular basis for the slower mobility of the small RNA fragments generated in the ATP-depleted lysate is not understood.

Wagner and Sun (Wagner and Sun, Nature, 391:744-745 (1998)) and Sharp (Sharp, Genes Dev., 13:139-41 (1999)) have speculated that the requirement for dsRNA in gene silencing by RNAi reflects the involvement of a dsRNA-specific adenosine deaminase in the process. dsRNA adenosine deaminases unwind dsRNA by converting adenosine to inosine, which does not base-pair with uracil. dsRNA adenosine deaminases function in the post-transcriptional editing of mRNA (for review see Bass, Trends Biochem. Sci., 22:157-62 (1997)). To test for the involvement of dsRNA adenosine deaminase in RNAi, the degree of conversion of adenosine to inosine in the 501 bp Rr-luc and 505 bp Pp-luc dsRNAs after incubation with Drosophila embryo lysate in a standard in vitro RNAi reaction was examined. Adenosine deamination in full-length dsRNA and the 21-23 nt RNA species was assessed by two-dimensional thin-layer chromatography. Inorganic phosphate (P_i) was produced by the degradation of mononucleotides by phosphatases that contaminate commercially available nuclease P1 (Auxilien et al., J. Mol. Biol., 262:437-458 (1996)). The degree of adenosine deamination in the 21-23 nt species was also determined. The full-length dsRNA radiolabeled with [^{32}P]-adenosine was incubated in the lysate, and both the full-length dsRNA and the 21-23 nt RNA products were purified from a denaturing acrylamide gel, cleaved to mononucleotides with nuclease P1, and analyzed by two-dimensional thin- layer chromatography.

A significant fraction of the adenosines in the full-length dsRNA were converted to inosine after 2 hours (3.1% and 5.6% conversion for Pp-luc and Rr-luc dsRNAs, respectively). In contrast, only 0.4% (Pp-dsRNA) or 0.7% (Rr-dsRNA) of the adenosines in the 21-23 nt species were deaminated. These data imply that fewer than 1 in 27 molecules of the 21-23 nt RNA species contain an inosine. Therefore, it is unlikely that dsRNA-dependent adenosine deamination within the 21-23 nt species is required for its production.

asRNA Generates a Small Amount of RNAi in vitro

When mRNA was ^{32}P-radiolabeled within the 5′-7-methyl-guanosine cap, stable 5′ decay products accumulated during the RNAi reaction. Such stable 5′decay products were observed for both the Pp-luc and Rr-luc mRNAs when they were incubated with their cognate dsRNAs. Previously, it was reported that efficient RNAi does not occur when asRNA is used in place of dsRNA (Tuschl et al., Genes Dev., 13:3191-7 (1999)). Nevertheless, mRNA was measurably less stable when incubated with asRNA than with buffer (Figures 8A and 8B). This was particularly evident for the Rr-luc mRNA: approximately 90% of the RNA remained intact after a 3-hour incubation in lysate, but only 50% when asRNA was added. Less than 5% remained when dsRNA was added. Interestingly, the decrease in mRNA stability caused by asRNA was accompanied by the formation of a small amount of the stable 5′-decay products characteristic of the RNAi reaction with dsRNA. This finding parallels the observation that a small amount of 21- 23 nt product formed from the asRNA when it was incubated with the mRNA (see above) and lends strength to the idea that asRNA can enter the RNAi pathway, albeit inefficiently.

mRNA Cleavage Sites Are Determined by the Sequence of the dsRNA

The sites of mRNA cleavage were examined using three different dsRNAs, 'A,' 'B,' and 'C,' displaced along the Rr-luc sequence by approximately 100 nts. Denaturing acrylamide-gel analysis of the stable, 5′-cleavage products produced after incubation of the Rr-luc mRNA for the indicated times with each of the three dsRNAs, 'A,' 'B,' and 'C,' or with buffer (Ø) was performed. The positions of these relative to the Rr-luc mRNA sequence are shown in Figure 9. Each of the three dsRNAs was incubated in a standard RNAi reaction with Rr-luc mRNA ^{32}P-radiolabeled within the 5′-cap. In the absence of dsRNA, no stable 5′-cleavage products were detected for the mRNA, even after 3 hours of incubation in lysate. In contrast, after a 20-minute incubation, each of the three dsRNAs produced a ladder of bands corresponding to a set of mRNA cleavage products characteristic for that particular dsRNA. For each dsRNA, the stable, 5′ mRNA cleavage products were restricted to the region of the Rr-luc mRNA that corresponded to the dsRNA

(Figures 9 and 10). For dsRNA 'A,' the lengths of the 5′- cleavage products ranged from 236 to just under ~750 nt; dsRNA 'A' spans nucleotides 233 to 729 of the Rr-luc mRNA. Incubation of the mRNA with dsRNA 'B' produced mRNA 5′-cleavage products ranging in length from 150 to ~600 nt; dsRNA 'B' spans nucleotides 143 to 644 of the mRNA. Finally, dsRNA 'C' produced mRNA cleavage products from 66 to ~500 nt in length. This dsRNA spans nucleotides 50 to 569 of the Rr-luc mRNA. Therefore, the dsRNA not only provides specificity for the RNAi reaction, selecting which mRNA from the total cellular mRNA pool will be degraded, but also determines the precise positions of cleavage along the mRNA sequence.

The mRNA Is Cleaved at 21-23 Nucleotide Intervals

To gain further insight into the mechanism of RNAi, the positions of several mRNA cleavage sites for each of the three dsRNAs were mapped (Figure 10). High resolution denaturing acrylamide-gel analysis of a subset of the 5′-cleavage products described above was performed. Remarkably, most of the cleavages occurred at 21-23 nt intervals (Figure 10). This spacing is especially striking in light of our observation that the dsRNA is processed to a 21-23 nt RNA species and the finding of Hamilton and Baulcombe that a 25 nt RNA correlates with post-transcriptional gene silencing in plants (Hamilton and Baulcombe, Science, 286:950-2 (1999)). Of the 16 cleavage sites we mapped (2 for dsRNA 'A,' 5 for dsRNA 'B,' and 9 for dsRNA 'C'), all but two reflect the 21-23 nt interval. One of the two exceptional cleavages was a weak cleavage site produced by dsRNA 'C' (indicated by an open blue circle in Figure 10). This cleavage occurred 32 nt 5′ to the next cleavage site. The other exception is particularly intriguing. After four cleavages spaced 21-23 nt apart, dsRNA 'C' caused cleavage of the mRNA just nine nt 3′ to the previous cleavage site (red arrowhead in Figure 10). This cleavage occurred in a run of seven uracil residues and appears to "reset" the ruler for cleavage; the next cleavage site was 21-23 nt 3′ to the exceptional site. The three subsequent cleavage sites that we mapped were also spaced 21-23 nt apart. Curiously, of the sixteen cleavage sites caused by the three different dsRNAs, fourteen occur at uracil residues. The

significance of this finding is not understood, but it suggests that mRNA cleavage is determined by a process which measures 21-23 nt intervals and which has a sequence preference for cleavage at uracil. Results show that the 21-23 nt RNA species produced by incubation of ~500 bp dsRNA in the lysate caused sequence-specific interference in vitro when isolated from an acrylamide gel and added to a new RNAi reaction in place of the full-length dsRNA.

A Model for dsRNA-directed mRNA Cleavage

Without wishing to be bound by theory, the biochemical data described herein, together with recent genetic experiments in C. elegans and Neurospora (Cogoni and Macino, Nature, 399:166-9 (1999); Grishok et al., Science, 287: 2494-7 (2000); Ketting et al., Cell, 99:133-41 (1999); Tabara et al., Cell, 99:123-32 (1999)), suggest a model for how dsRNA targets mRNA for destruction (Figure 11). In this model, the dsRNA is first cleaved to 21-23 nt long fragments in a process likely to involve genes such as the C. elegans loci rde-1 and rde-4. The resulting fragments, probably as short asRNAs bound by RNAi-specific proteins, would then pair with the mRNA and recruit a nuclease that cleaves the mRNA. Alternatively, strand exchange could occur in a protein-RNA complex that transiently holds a 21-23 nt dsRNA fragment close to the mRNA. Separation of the two strands of the dsRNA following fragmentation might be assisted by an ATP-dependent RNA helicase, explaining the observed ATP enhancement of 21-23 nt RNA production.

It is likely that each small RNA fragment produces one, or at most two, cleavages in the mRNA, perhaps at the 5' or 3' ends of the 21-23 nt fragment. The small RNAs may be amplified by an RNA-directed RNA polymerase such as that encoded by the ego-1 gene in C. elegans (Smardon et al., Current Biology, 10:169-178 (2000)) or the qde-1 gene in Neurospora (Cogoni and Macino, Nature, 399:166-9 (1999)), producing long-lasting post-transcriptional gene silencing in the absence of the dsRNA that initiated the RNAi effect. Heritable RNAi in C. elegans requires the rde-1 and rde-4 genes to initiate, but not to persist in subsequent generations. The rde-2, rde- 3, and mut-7 genes in C. elegans are required in the tissue where RNAi occurs, but are not required for initiation of heritable RNAi

(Grishok et al., Science, in press 2000). These 'effector' genes (Grishok et al., Science, in press 2000) are likely to encode proteins functioning in the actual selection of mRNA targets and in their subsequent cleavage. ATP may be required at any of a number of steps during RNAi, including complex formation on the dsRNA, strand dissociation during or after dsRNA cleavage, pairing of the 21-23 nt RNAs with the target mRNA, mRNA cleavage, and recycling of the targeting complex. Testing these ideas with the in vitro RNAi system will be an important challenge for the future. Some genes involved in RNAi are also important for transposon silencing and co-suppresion. Co-suppression is a broad biological phenomenon spanning plants, insects and perhaps humans. The most likely mechanism in Drosophila melanogaster is transcriptional silencing (Pal-Bhanra et al, Cell 99: 35-36. Thus, 21-23 nt fragments are likely to be involved in transcriptional control, as well as in post-transcriptional cotrol.

Example 3 Isolated 21-23 mers caused sequence-specific interference when added to a new RNAi reaction

Isolation of 21-23 nt fragments from incubation reaction of 500 bp dsRNA in lysate.

Double-stranded RNA (500 bp from) was incubated at 10 nM concentration in Drosophila embryo lysate for 3 h at 25° C under standard conditions as described herein. After deproteinization of the sample, the 21-23 nt reaction products were separated from unprocessed dsRNA by denaturing polyacrylamide (15%) gel electrophoresis. For detection of the non-radiolabeled 21-23 nt fragments, an incubation reaction with radiolabeled dsRNA was loaded in a separate lane of the same gel. Gel slices containing the non-radioactive 21-23 nt fragments were cut out and the 21-23 nt fragments were eluted from the gel slices at 4° C overnight in 0.4 ml 0.3 M NaCl. The RNA was recovered from the supernatant by ethanol precipitation and centrifugation. The RNA pellet was dissolved in 10 µl of lysis buffer. As control, gel slices slightly above and below the 21-23 nt band were also cut out and subjected to the same elution and precipitation procedures. Also, a non-incubated dsRNA loaded on the 15% gel and a gel slice corresponding to 21-23 nt fragments was cut out and eluted. All pellets from the control experiments were

dissolved in 10 µl lysis buffer. The losses of RNA during recovery from gel slices by elution are approx. 50%.

Incubation of purified 21-23 nt fragments in a translation-based RNAi assay

1 µl of the eluted 21-23 mer or control RNA solution was used for a standard 10 µl RNAi incubation reaction (see above). The 21-23 mers were preincubated in the lysate containing reaction mixture for 10 or 30 min before the addition of the target and control mRNA. During pre-incubation, proteins involved in RNA interference may re- associate with the 21-23 mers due to a specific signal present on these RNAs. The incubation was continued for another hour to allow translation of the target and control mRNAs. The reaction was quenched by the addition of passive lysis buffer (Promega), and luciferase activity was measured. The RNA interference is the expressed as the ratio of target to control luciferase activity normalized by an RNA-free buffer control. Specific suppression of the target gene was observed with either 10 or 30 minutes pre- incubation. The suppression was reproducible and reduced the relative ratio of target to control by 2-3 fold. None of the RNA fragments isolated as controls showed specific interference. For comparison, incubation of 5 nM 500 bp dsRNA (10 min pre- incubation) affects the relative ratio of control to target gene approx. 30-fold.

Stability of isolated 21-23 nt fragments in a new lysate incubation reaction.

Consistent with the observation of RNAi mediated by purified 21-23 nt RNA fragment, it was found that 35% of the input 21-23 nt RNA persists for more than 3 h in such an incubation reaction. This suggests that cellular factors associate with the deproteinized 21-23 nt fragments and reconstitute a functional mRNA-degrading particle. Signals connected with these 21-23 nt fragments, or their possible double stranded nature or specific lengths are likely responsible for this observation. The 21-23 nt fragments have a terminal 3' hydroxyl group, as evidenced by altered mobility on a sequencing gel following periodate treatment and beta-elimination.

Example 4 21-23-mers purified by non-denaturing methods caused sequence-specific interference when added to a new RNAi reaction.

Fifty nanomolar double-stranded RNA (501 bp Rr-luc dsRNA, as described in example 1) was incubated in a 1 ml in vitro reaction with lysate at 25°C (see example 1). The reaction was then stopped by the addition of an equal volume of 2x PK buffer (see example 1) and proteinase K was added to a final concentration of 1.8 µg/µl. The reaction was incubated for an additional 1 h at 25°C, phenol extracted, and then the RNAs were precipitated with 3 volumes of ethanol. The ethanol precipitate was collected by centrifugation, and the pellet was resuspended in 100 µl of lysis buffer and applied to a Superdex HR 200 10/30 gel filtration column (Pharmacia) run in lysis buffer at 0.75 ml/min. 200 µl fractions were collected from the column. Twenty µl of 3 M sodium acetate and 20 µg glycogen was added to each fraction, and the RNA was recovered by precipitation with 3 volumes of ethanol. The precipitates were resuspended in 30 µl of lysis buffer. Column profiles following the fractionation of 32P-labeled input RNA are shown in Figure 13A.

One microliter of each resuspended fraction was tested in a 10 µl standard in vitro RNAi reaction (see example 1). This procedure yields a concentration of RNA in the in vitro RNAi reaction that is approximately equal to the concentration of that RNA species in the original reaction prior to loading on the column. The fractions were preincubated in the lysate containing reaction mixture for 30 min before the addition of 10 nM Rr-luc mRNA target and 10 nM Pp-luc control mRNA. During pre-incubation, proteins involved in RNA interference may re-associate with the 21-23-mers due to a specific signal present on these RNAs. The incubation was continued for another three hours to allow translation of the target and control mRNAs. The reaction was quenched by the addition of passive lysis buffer (Promega), and luciferase activity was measured. The suppression of Rr-luc mRNA target expression by the purified 21-23 nt fragments was reproducible and reduced the relative ratio of target to control by >30-fold, an amount comparable to a 50 nM 500 bp dsRNA control. Suppression of target mRNA expression was specific: little or no effect on the expression of the Pp-luc mRNA control was observed.

The data show that the both the fractions containing uncleaved dsRNA (fractions 3 - 5) or long, partially cleaved dsRNA (fractions 7 - 13) and the fractions containing the fully processed 21-23 nt siRNAs (fractions 41 - 50) mediate effective RNA interference in vitro (Figure 13B). Suppression of target mRNA expression was specific: little or no effect on the expression of the Pp-luc mRNA control was observed (Figure 13C). These data, together with those in the earlier examples, demonstrate that the 21-23 nt siRNAs are (1) true intermediates in the RNAi pathway and (2) effective mediators of RNA interference in vitro.

Example 5 21-nucleotide siRNA duplexes mediate RNA interference in human tissue cultures

Methods

RNA preparation

21 nt RNAs were chemically synthesized using Expedite RNA phosphoramidites and thymidine phosphoramidite (Proligo, Germany). Synthetic oligonucleotides were deprotected and gel-purified (Elbashir, S.M., Lendeckel, W. & Tuschl, T., *Genes & Dev. 15*, 188-200 (2001)), followed by Sep-Pak C18 cartridge (Waters, Milford, MA, USA) purification (Tuschl, t., *et al., Biochemistry, 32:*11658-11668 (1993)). The siRNA sequences targeting GL2 (Acc. X65324) and GL3 luciferase (Acc. U47296) corresponded to the coding regions 153-173 relative to the first nucleotide of the start codon, siRNAs targeting RL (Acc. AF025846) corresponded to region 119-129 after the start codon. Longer RNAs were transcribed with T7 RNA polymerase from PCR products, followed by gel and Sep-Pak purification. The 49 and 484 bp GL2 or GL3 dsRNAs corresponded to position 113-161 and 113-596, respectively, relative to the start of translation; the 50 and 501 bp RL dsRNAs corresponded to position 118-167 and 118-618, respectively. PCR templates for dsRNA synthesis targeting humanized GFP (hG) were amplified from pAD3 (Kehlenbach, R.H., *et al., J. Cell Biol., 141:*863-874 (1998)), whereby 50 and 501 bp hG dsRNA corresponded to position 118-167 and 118-618, respectively, to the start codon.

For annealing of siRNAs, 20 μM single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C followed by 1 h at 37 °C. The 37 °C incubation step was extended overnight for the 50 and 500 bp dsRNAs, and these annealing reactions were performed at 8.4 μM and 0.84 μM strand concentrations, respectively.

Cell culture

S2 cells were propagated in Schneider's *Drosophila* medium (Life Technologies) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 25 °C. 293, NIH/3T3, HeLa S3, COS-7 cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were regularly passaged to maintain exponential growth. 24 h before transfection at approx. 80% confluency, mammalian cells were trypsinized and diluted 1:5 with fresh medium without antibiotics (1-3 × 10^5 cells/ml) and transferred to 24-well plates (500 μl/well). S2 cells were not trypsinized before splitting. Transfection was carried out with Lipofectamine 2000 reagent (Life Technologies) as described by the manufacturer for adherent cell lines. Per well, 1.0 μg pGL2-Control (Promega) or pGL3-Control (Promega), 0.1 μg pRL-TK (Promega), and 0.28 μg siRNA duplex or dsRNA, formulated into liposomes, were applied; the final volume was 600 μl per well. Cells were incubated 20 h after transfection and appeared healthy thereafter. Luciferase expression was subsequently monitored with the Dual luciferase assay (Promega). Transfection efficiencies were determined by fluorescence microscopy for mammalian cell lines after co-transfection of 1.1 μg hGFP-encoding pAD3[22] and 0.28 μg invGL2 siRNA, and were 70-90%. Reporter plasmids were amplified in XL-1 Blue (Strategene) and purified using the Qiagen EndoFree Maxi Plasmid Kit.

Results

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded

RNA (dsRNA) homologous in sequence to the silenced gene (Fire, A., *Trends Genet., 15:*358-363 (1999); Sharp, P.A. & Zamore, P.D., *Science, 287:*2431-2433 (2000); Sijen, T. & Kooter, J.M., *Bioessays, 22:*520-531 (2000); Bass, B.L., *Cell, 101:*235-238 (2000); Hammond, S.M., *et al., Nat. Rev. Genet., 2:*110-119 (2001)). The mediators of sequence-specific mRNA degradation are 21 and 22 nt small interfering RNAs (siRNAs) generated by RNase III cleavage from longer dsRNAs[6-10] (Hamilton, A.J. &Baulcombe, D.C., *Science, 286:*950-952 (1999); Hammond, S.M., *et al., Nature, 404:*293-296 (2000); Zamore, P.D., *et al., Cell, 101:*25-33 (2000); Bernstein, E., *et al., Naature, 409:*363-366 (2001); Elbashir, S.M., *et al., Genes & Dev., 15:*188-200 (2001)). As shown herein, 21 nt siRNA duplexes are able to specifically suppress reporter gene expression in multiple mammalian tissue cultures, including human embryonic kidney (293) and HeLa cells. In contrast to 50 or 500 bp dsRNAs, siRNAs do not activate the interferon response. These results indicate that siRNA duplexes are a general tool for sequence-specific inactivation of gene function in mammalian cells.

Base-paired 21 and 22 nt siRNAs with overhanging 3' ends mediate efficient sequence-specific mRNA degradation in lysates prepared from *D. melanogaster* embryos (Elbashir, S.M., *et al., Genes & Dev., 15:*188-200 (2001)). To test whether siRNAs are also capable of mediating RNAi in tissue culture, 21 nt siRNA duplexes with symmetric 2 nt 3' overhangs directed against reporter genes coding for sea pansy (*Renilla reniformis*) and two sequence variants of firefly (*Photinus pyralis,* GL2 and GL3) luciferases (Figures 14A, 14B) were constructed. The siRNA duplexes were co-transfected with the reporter plasmid combinations pGL2/pRL or pGL3/pRL, into *D. melanogaster Schneider S2* cells or mammalian cells using cationic liposomes. Luciferase activities were determined 20 h after transfection. In all cell lines tested, specific reduction of the expression of the reporter genes in the presence of cognate siRNA duplexes was observed (Figures 15A-15J). Remarkably, the absolute luciferase expression levels were unaffected by non-cognate siRNAs, indicating the absence of harmful side effects by 21 nt RNA duplexes (e.g. Figures 16A-16D, for HeLa cells). In *D. melanogaster* S2 cells (Figures 15A, 15B), the specific inhibition of luciferases was complete, and similar to results previously

obtained for longer dsRNAs (Hammond, S.M., *et al., Nature, 404:*293-296 (2000); Caplen, N.J., *et al., Gene, 252:*95-105 (2000); Clemens, M & Williams, B., *Cell, 13:*565-572 (1978); Ui-Tei, K., *et al., FEBS Letters, 479:*79-82 (2000)). In mammalian cells, where the reporter genes were 50- to 100-fold stronger expressed, the specific suppression was less complete (Figures 15C-15J). GL2 expression was reduced 3- to 12-fold, GL3 expression 9- to 25-fold, and RL expression 1- to 3-fold, in response to the cognate siRNAs. For 293 cells, targeting of RL luciferase by RL siRNAs was ineffective, although GL2 and GL3 targets responded specifically (Figures 15I, 15J). It is likely that the lack of reduction of RL expression in 293 cells is due to its 5- to 20-fold higher expression compared to any other mammalian cell line tested and/or to limited accessibility of the target sequence due to RNA secondary structure or associated proteins. Nevertheless, specific targeting of GL2 and GL3 luciferase by the cognate siRNA duplexes indicated that RNAi is also functioning in 293 cells.

The 2 nt 3' overhang in all siRNA duplexes, except for uGL2, was composed of (2'-deoxy) thymidine. Substitution of uridine by thymidine in the 3' overhang was well tolerated in the *D. melanogaster* in vitro system, and the sequence of the overhang was uncritical for target recognition (Elbashir, S.M., *et al., Genes & Dev., 15:*188-200 (2001)). The thymidine overhang was chosen, because it is supposed to enhance nuclease resistance of siRNAs in the tissue culture medium and within transfected cells. Indeed, the thymidine-modified GL2 siRNA was slightly more potent than the unmodified uGL2 siRNA in all cell lines tested (Figures 15A, 15C, 15E, 15G, 15I). It is conceivable that further modifications of the 3' overhanging nucleotides will provide additional benefits to the delivery and stability of siRNA duplexes.

In co-transfection experiments, 25 nM siRNA duplexes with respect to the final volume of tissue culture medium were used (Figures 15A-15J, 16A-16F). Increasing the siRNA concentration to 100 nM did not enhance the specific silencing effects, but started to affect transfection efficiencies due to competition for liposome encapsulation between plasmid DNA and siRNA. Decreasing the siRNA concentration to 1.5 nM did not reduce the specific silencing effect, even though the

siRNAs were now only 2- to 20-fold more concentrated than the DNA plasmids. This indicates that siRNAs are extraordinarily powerful reagents for mediating gene silencing, and that siRNAs are effective at concentrations that are several orders of magnitude below the concentrations applied in conventional antisense or ribozyme gene targeting experiments.

In order to monitor the effect of longer dsRNAs on mammalian cells, 50 and 500 bp dsRNAs cognate to the reporter genes were prepared. As non-specific control, dsRNAs from humanized GFP (hG) (Kehlenbach, R.H., *et al., J. Cell Biol., 141:*863-874 (1998)) was used. When dsRNAs were co-transfected, in identical amounts (not concentrations) to the siRNA duplexes, the reporter gene expression was strongly and unspecifically reduced. This effect is illustrated for HeLa cells as a representative example (Figures 16A-16D). The absolute luciferase activities were decreased unspecifically 10- to 20-fold by 50 bp dsRNA, and 20- to 200-fold by 500 bp dsRNA co-transfection, respectively. Similar unspecific effects were observed for COS-7 and NIH/3T3 cells. For 293 cells, a 10- to 20-fold unspecific reduction was observed only for 500 bp dsRNAs. Unspecific reduction in reporter gene expression by dsRNA > 30 bp was expected as part of the interferon response (Matthews, M., Interactions between viruses and the cellular machinery for protein synthesis in *Translational Control* (eds., Hershey, J., Matthews,M. & Sonenberg, N.) 505-548 (Cold Spring Harbor Laboratory Press, Plainview, NY; 1996); Kumar, M. & Carmichael, G.G., *Microbiol. Mol. Biol. Rev., 62:*1415-1434 (1998); Stark, G.R., *et al., Annu. Rev. Biochem., 67:*227-264 (1998)). Surprisingly, despite the strong unspecific decrease in reporter gene expression, additional sequence-specific, dsRNA-mediated silencing were reproducibly detected. The specific silencing effects, however, were only apparent when the relative reporter gene activities were normalized to the hG dsRNA controls (Figures 16E, 16F). A 2- to 10-fold specific reduction in response to cognate dsRNA was observed, also in the other three mammalian cell lines tested. Specific silencing effects with dsRNAs (356-1662 bp) were previously reported in CHO-K1 cells, but the amounts of dsRNA required to detect a 2- to 4-fold specific reduction were about 20-fold higher than in our experiments (Ui-Tei, K., *et al., FEBS Letters, 479:*79-82 (2000)). Also, CHO-K1

cells appear to be deficient in the interferon response. In another report, 293, NIH/3T3, and BHK-21 cells were tested for RNAi using luciferase/lacZ reporter combinations and 829 bp specific lacZ or 717 bp unspecific GFP dsRNA (Caplen, N.J., *et al., Gene, 252*:95-105 (2000)). The failure of detecting RNAi in this case is likely due to the less sensitive luciferase/lacZ reporter assay and the length differences of target and control dsRNA. Taken together, the results described herein indicate that RNAi is active in mammalian cells, but that the silencing effect is difficult to detect if the interferon system is activated by dsRNA >30 bp.

The mechanism of the 21 nt siRNA-mediated interference process in mammalian cells remains to be uncovered, and silencing may occur post-transcriptional and/or transcriptional. In *D. melanogaster* lysate, siRNA duplexes mediate post-transcriptional gene silencing by reconstitution of a siRNA-protein complexes (siRNPs), which are guiding mRNA recognition and targeted cleavage (Hammond, S.M., *et al., Nature, 404*:293-296 (2000); Zamore, P.D., *et al., Cell, 101*:25-33 (2000); Elbashir, S.M., *et al., Genes & Dev., 15*:188-200 (2001)). In plants, dsRNA-mediated post-transcriptional silencing has also been linked to RNA-directed DNA methylation, which may also be directed by 21 nt siRNAs (Wassenegger, M., *Plant Mol. Biol. 43*:203-220 (2000); Finnegan, E.J., *et al., Curr. Biol., 11*:R99-R102 (2000)). Methylation of promoter regions can lead to transcriptional silencing (Metter, M.F., *et al., EMBO J., 19*:5194-5201 (2000)), but methylation in coding sequences must not (Wang, M.-B., *RNA, 7*:16-28 (2001)). DNA methylation and transcriptional silencing in mammals are well-documented processes (Kass, S.U., *et al., Trends Genet., 13*:444-449 (1997); Razin, A., *EMBO J, 17*:4905-4908 (1998)), yet they have not been linked to post-transcriptional silencing. Methylation in mammals is predominantly directed towards CpG residues. Because there is no CpG in the RL siRNA, but RL siRNA mediates specific silencing in mammalian tissue culture, it is unlikely that DNA methylation is critical for our observed silencing process. In summary, described herein, is siRNA-mediated gene silencing in mammalian cells. The use of 21 nt siRNAs holds great promise for inactivation of gene function in human tissue culture and the development of gene-specific therapeutics.

While this invention has been particularly shown and described with reference to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims

CLAIMS

What is claimed is:

1. Isolated RNA of from about 21 to about 23 nucleotides that mediates RNA interference of an mRNA to which it corresponds.

2. Isolated RNA of claim 1 that comprises a terminal 3' hydroxyl group.

3. Isolated RNA of claim 1 which is chemically synthesized RNA or an analog of a naturally occurring RNA.

4. An analog of isolated RNA of claim 1, wherein the analog differs from the RNA of claim 1 by the addition, deletion, substitution or alteration of one or more nucleotides.

5. Isolated RNA of from about 21 to about 23 nucleotides that inactivates a corresponding gene by transcriptional silencing.

6. A soluble extract that mediates RNA interference.

7. The soluble extract of Claim 6, wherein the extract is derived from Drosophila embryos.

8. The soluble extract of Claim 7 wherein the extract is derived from syncytial blastoderm Drosophila embryos.

9. A method of producing RNA of from about 21 to about 23 nucleotides in length comprising:

(a) combining double-stranded RNA with a soluble extract that mediates RNA interference, thereby producing a combination; and

(b) maintaining the combination of a) under conditions in which the double-stranded RNA is processed to RNA of from about 21 to about 23 nucleotides in length.

10. The method of Claim 9, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

11. The method of Claim 9 further comprising isolating the RNA of from about 21 to about 23 nucleotides from the combination.

12. RNA of about 21 to about 23 nucleotides produced by the method of Claim 9.

13. A method of producing RNA of from about 21 to about 23 nucleotides in length that mediates RNA interference of mRNA of a gene to be degraded, comprising:

(a) combining double-stranded RNA that corresponds to a sequence of the gene to be degraded with a soluble extract that mediates RNA interference, thereby producing a combination; and

(b) maintaining the combination of (a) under conditions under which the double-stranded RNA is processed to RNA of from about 21 to about 23 nucleotides that mediates RNA interference of the mRNA of the gene to be degraded, thereby producing RNA of from about 21 to about 23 nucleotides that mediates RNA interference of the mRNA.

14. The method of Claim 13, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

15. The method of Claim 13 further comprising isolating RNA of from about 21 to about 23 nucleotides from the combination.

16. Isolated RNA of from about 21 to about 23 nucleotides produced by the method of Claim 15.

17. A method of mediating RNA interference of mRNA of a gene in a cell or organism comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides which targets the mRNA of the gene for degradation into the cell or organism;

(b) maintaining the cell or organism produced in (a) under conditions under which degradation of the mRNA occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism.

18. The method of Claim17 wherein the RNA of (a) is a chemically synthesized RNA or an analog of naturally occurring RNA.

19. The method of Claim 17, wherein the gene encodes a cellular mRNA or a viral mRNA.

20. A method of mediating RNA interference of mRNA of a gene in a cell or organism in which RNA interference occurs, comprising:

(a) combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract that mediates RNA interference, thereby producing a combination;

(b) maintaining the combination produced in (a) under conditions under which the double- stranded RNA is processed to RNA of from about 21 to about 23 nucleotides, thereby producing RNA of from about 21 to about 23 nucleotides;

(c) isolating RNA of from about 21 to about 23 nucleotides produced in (b);

(d) introducing RNA isolated in (c) into the cell or organism; and

(e) maintaining the cell or organism produced in (d) under conditions under which degradation of mRNA of the gene occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism.

21. The method of Claim 20, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

22. The method of Claim 20, wherein the RNA is isolated using gel electrophoresis.

23. A method of mediating RNA interference of mRNA of a gene in a cell or organism in which RNA interference occurs, comprising: (a) introducing into the cell or organism RNA of from about 21 to about 23 nucleotides that mediates RNA interference of mRNA of the gene, thereby producing a cell or organism that contains the RNA and (b) maintaining the cell or organism that contains the RNA under conditions under which RNA interference occurs, thereby mediating RNA interference of mRNA of the gene in the cell or organism.

24. The method of claim 23, wherein the RNA of from about 21 to about 23 nucleotides is chemically synthesized RNA or an analog of RNA that mediates RNA interference.

25. The method of Claim 23, wherein the gene encodes a cellular mRNA or a viral mRNA.

26. A knockdown cell or organism generated by the method of claim 23.

27. The knockdown cell or organism of claim 26, wherein the cell or organism mimics a disease.

28. A method of examining the function of a gene in a cell or organism comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides that targets mRNA of the gene for degradation into the cell or organism, thereby producing a test cell or test organism;

(b) maintaining the test cell or test organism under conditions under which degradation of mRNA of the gene occurs, thereby producing a test cell or test organism in which mRNA of the gene is degraded; and

(c) observing the phenotype of the test cell or test organism produced in (b) and, optionally, comparing the phenotype observed to that of an appropriate control cell or control organism, thereby providing information about the function of the gene.

29. The method of Claim 28 wherein the RNA introduced in (a) is chemically synthesized or an analog of RNA that mediates RNA interference.

30. A method of examining the function of a gene in a cell or organism comprising

(a) combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract that mediates RNA interference, thereby producing a combination;

(b) maintaining the combination produced in (a) under conditions under which the double- stranded RNA is processed to RNA of about 21 to about 23 nucleotides, whereby RNA of about 21 to about 23 nucleotides is produced;

(c) isolating RNA of about 21 to about 23 nucleotides produced in (b);

(d) introducing the RNA isolated in (c) into the cell or organism, thereby producing a test cell or test organism;

(e) maintaining the test cell or test organism under conditions under which degradation of mRNA of the gene occurs, thereby producing a test cell or test organism in which mRNA of the gene is degraded; and

(f) observing the phenotype of the test cell or test organism produced in (e) and, optionally, comparing the phenotype observed to that of an appropriate control, thereby providing information about the function of the gene.

31. The method of claim 30, wherein the RNA comprises a terminal 3' hydroxyl group.

32. The method of claim 30, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

33. The method of claim 30, wherein the RNA is isolated using gel electrophoresis.

34. A composition comprising biochemical components of a Drosophila cell that process dsRNA to RNA of about 21 to about 23 nucleotides and a suitable carrier.

35. A composition comprising biochemical components of a cell that target mRNA of a gene to be degraded by RNA of about 21 to about 23 nucleotides.

36. A method of treating a disease or condition associated with the presence of a protein in an individual comprising administering to the individual RNA of from about 21 to about 23 nucleotides that targets the mRNA of the protein for degradation.

37. The method of claim 36 wherein RNA of from about 21 to about 23 nucleotides is chemically synthesized or an analog of RNA that mediates RNA interference.

38. A method of assessing whether an agent acts on a gene product comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides which targets the mRNA of the gene for degradation into a cell or organism;

(b) maintaining the cell or organism of (a) under conditions in which degradation of the mRNA occurs,

(c) introducing the agent into the cell or organism of (b); and

(d) determining whether the agent has an effect on the cell or organism, wherein if the agent has no effect on the cell or organism then the agent acts on the gene product or on a biological pathway that involves the gene product.

39. The method of claim 38, wherein the RNA of from about 21 to about 23 nucleotides is chemically synthesized or an analog of RNA that mediates RNA interference.

40. A method of assessing whether a gene product is a suitable target for drug discovery comprising:

(a) introducing RNA of from about 21 to about 23 nucleotides which targets the mRNA of the gene for degradation into a cell or organism;

(b) maintaining the cell or organism of (a) under conditions in which degradation of the mRNA occurs resulting in decreased expression of the gene; and

(c) determining the effect of the decreased expression of the gene on the cell or organism, wherein if decreased expression has an effect, then the gene product is a target for drug discovery.

41. The method of claim 40, wherein the RNA of from about 21 to about 23 nucleotides is synthetic RNA or an analog of RNA that mediates RNA interference.

42. A gene identified by the sequencing of endogenous 21 to 23 nucleotide RNA molecules that mediate RNA interference.

43. A pharmaceutical composition comprising RNA of from about 21 to about 23 nucleotides that mediates RNA interference and an appropriate carrier.

44. A method of producing knockdown cells, comprising introducing into cells in which a gene is to be knocked down RNA of about 21 to about 23 nt that targets the mRNA corresponding to the gene and maintaining the resulting cells under conditions under which RNAi occurs, resulting in degradation of the mRNA of the gene, thereby producing knockdown cells.

45. The method of claim 44, wherein the RNA of about 21 to about 23 nucleotides is synthetic RNA or an analog of RNA that mediates RNA interference.

46. A method of identifying target sites within mRNA that are efficiently cleaved by the RNAi process, comprising combining dsRNA corresponding to a sequence of a gene to be degraded, labeled mRNA corresponding to the gene and a soluble extract that mediates RNA interference, thereby producing a combination; maintaining the combination under conditions under which the dsRNA is degraded and identifying sites in the mRNA that are efficiently cleaved.

47. A method of identifying 21-23 nt RNAs that efficiently mediate RNAi, wherein said 21-23 nt RNAs span the target sites identified within the mRNA by the method of claim 46.

48. RNA of claim 16, isolated using gel electrophoresis.

49. RNA of claim 16, isolated using non-denaturing methods.

50. RNA of claim 16, isolated using non-denaturing column chromatography.

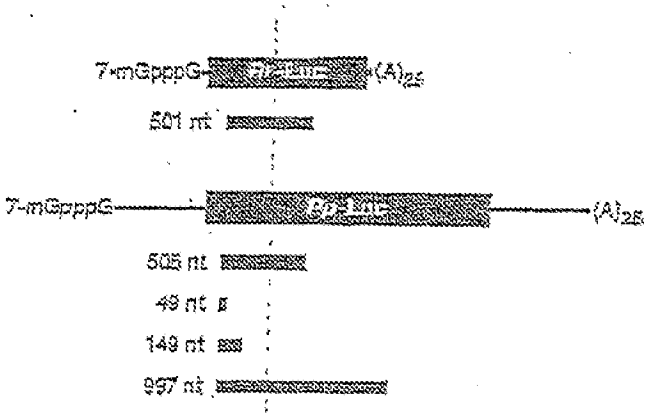


Figure 1

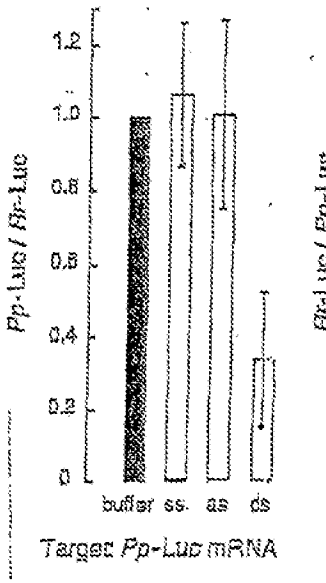


Figure 2A

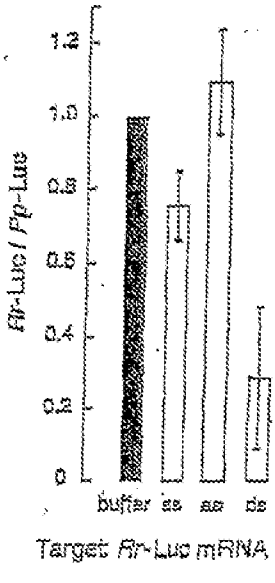


Figure 2B

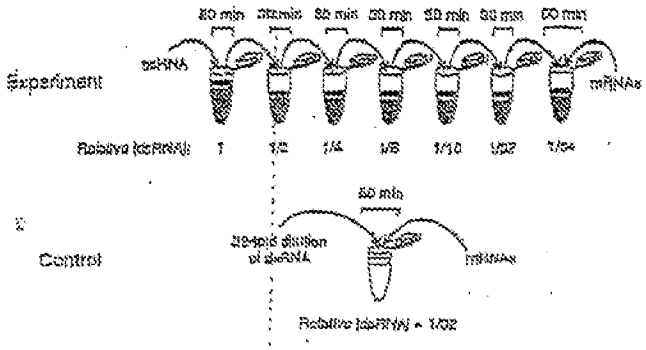


Figure 3A

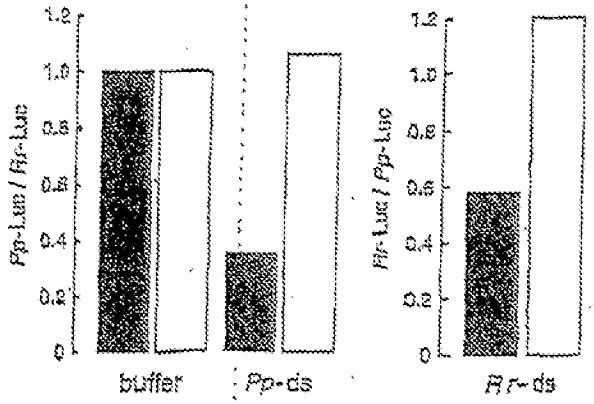


Figure 3B

Figure 3C

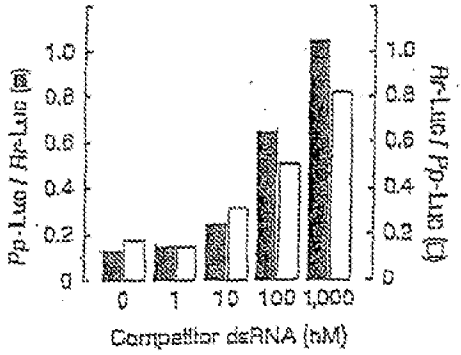


Figure 4

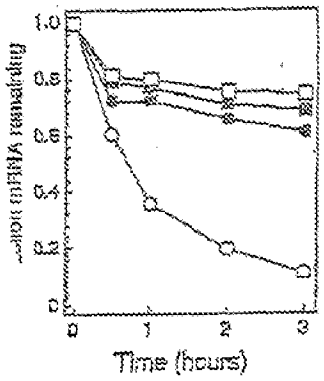


Figure 5A

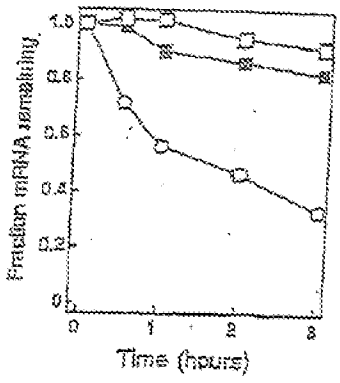


Figure 5B

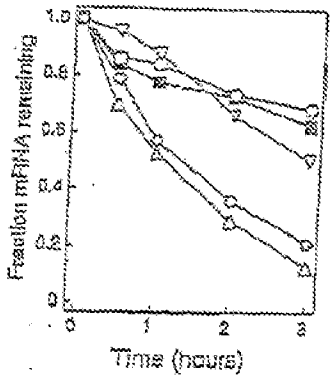


Figure 5C

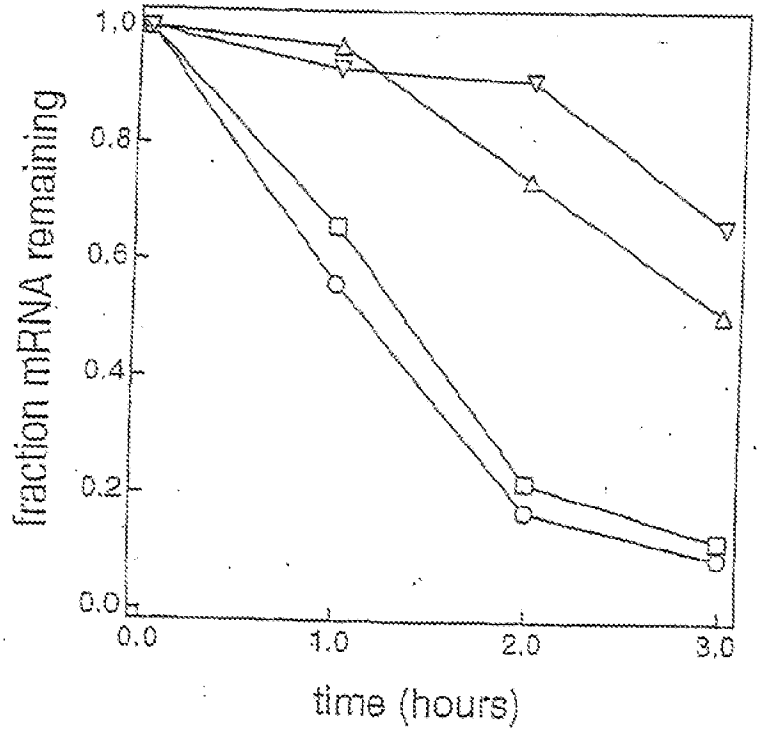


Figure 6

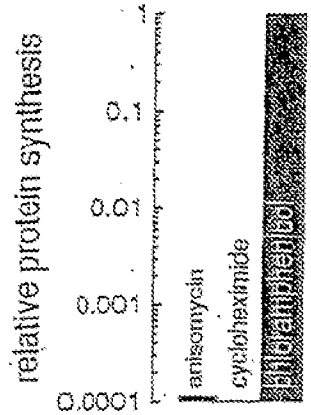


Figure 7A

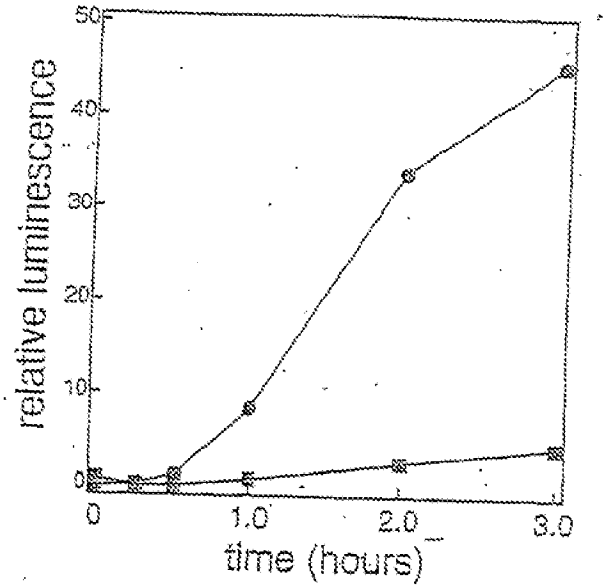


Figure 7B

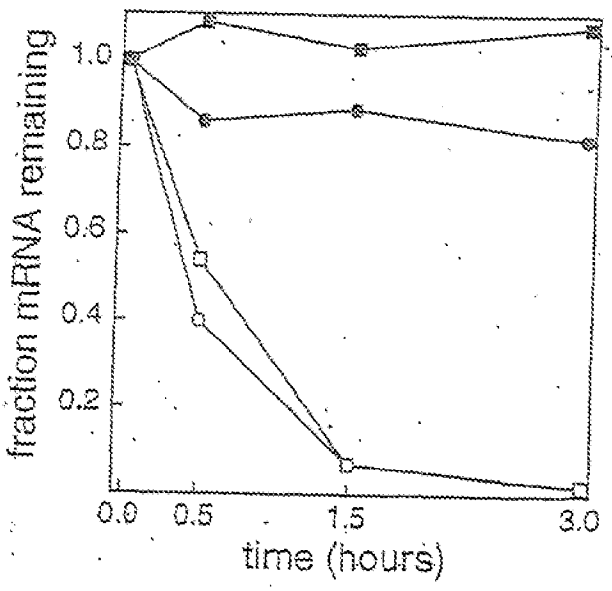


Figure 7C

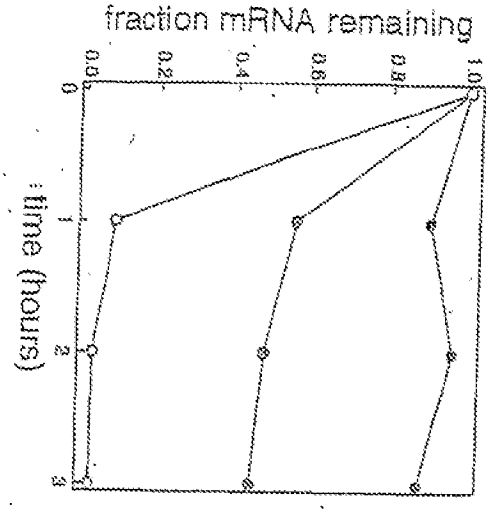


Figure 8A

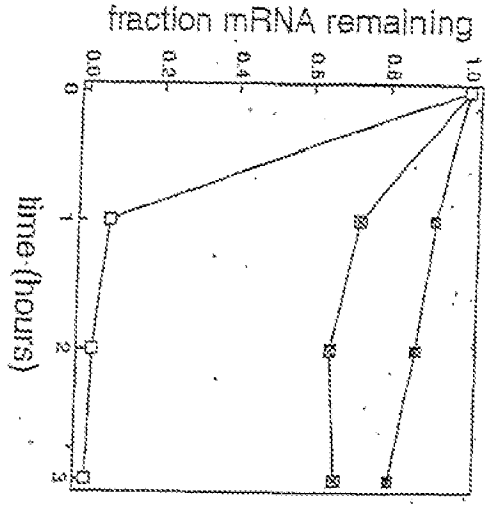


Figure 8B

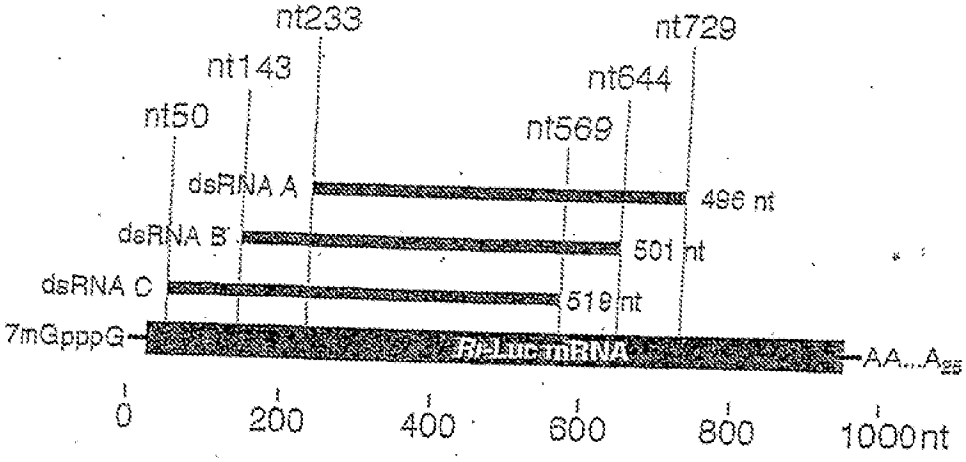
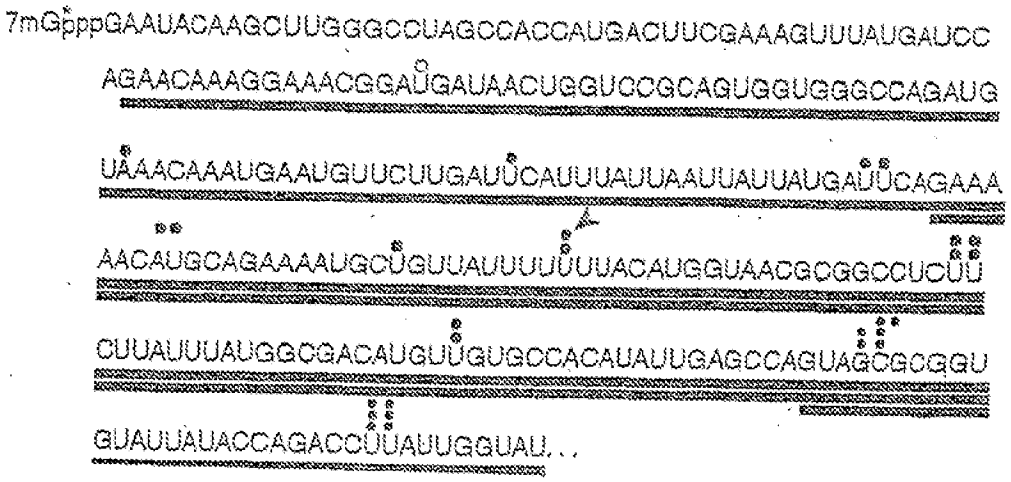


Figure 9

Figure 10



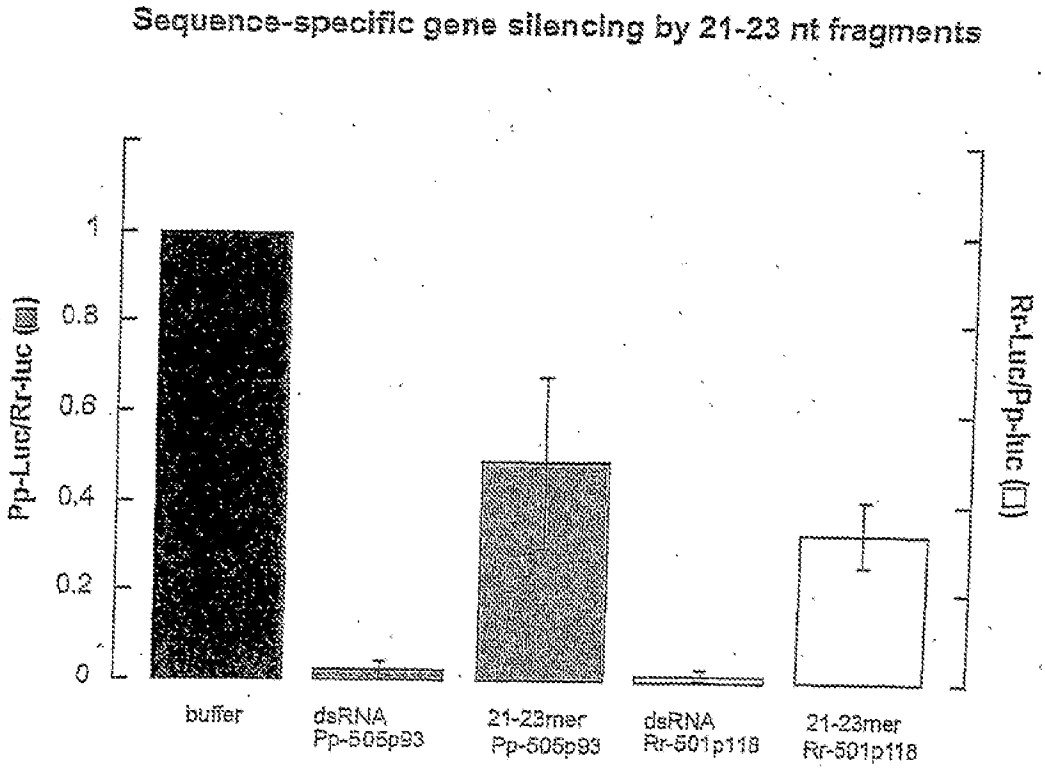


Figure 12

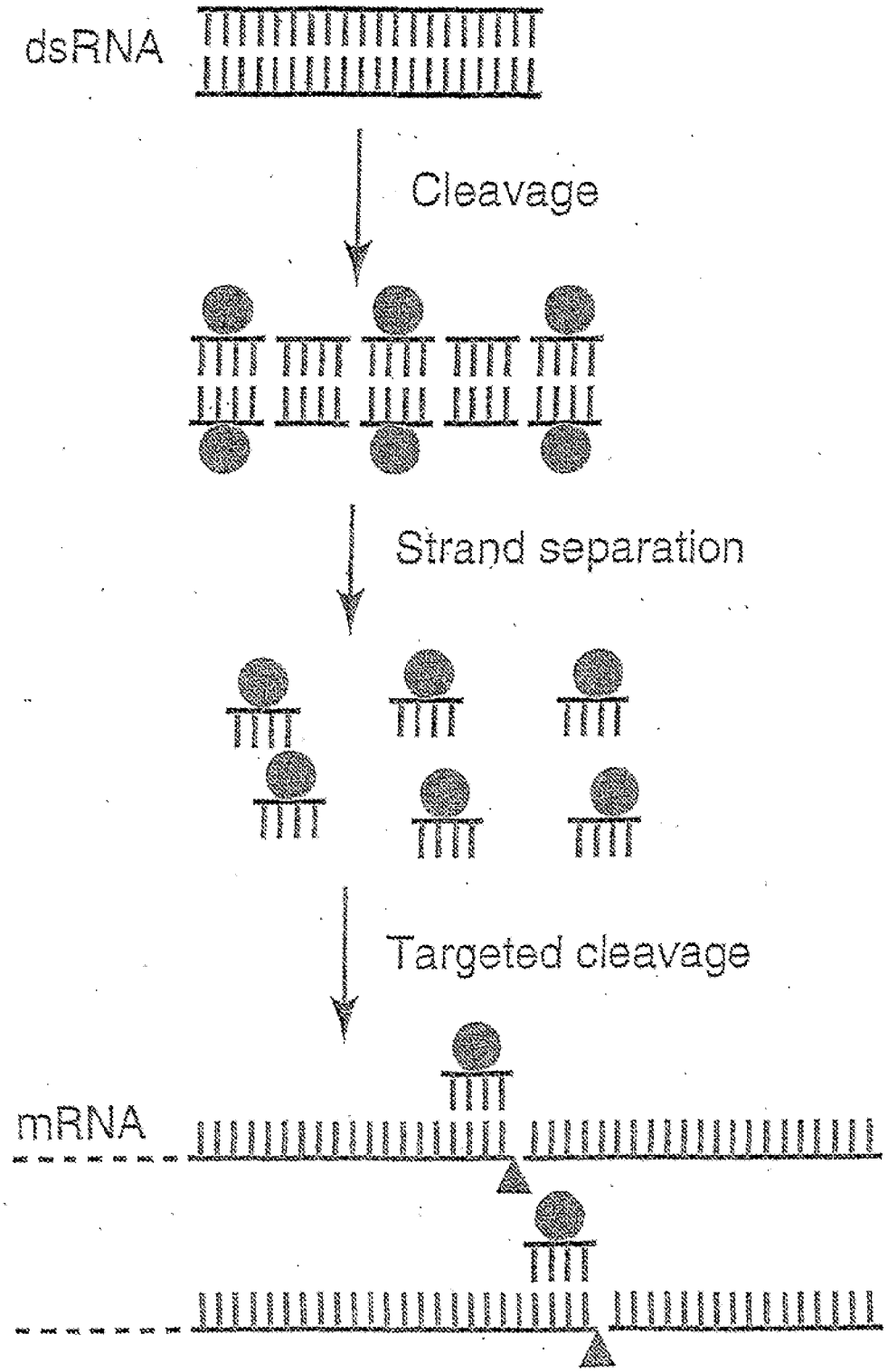
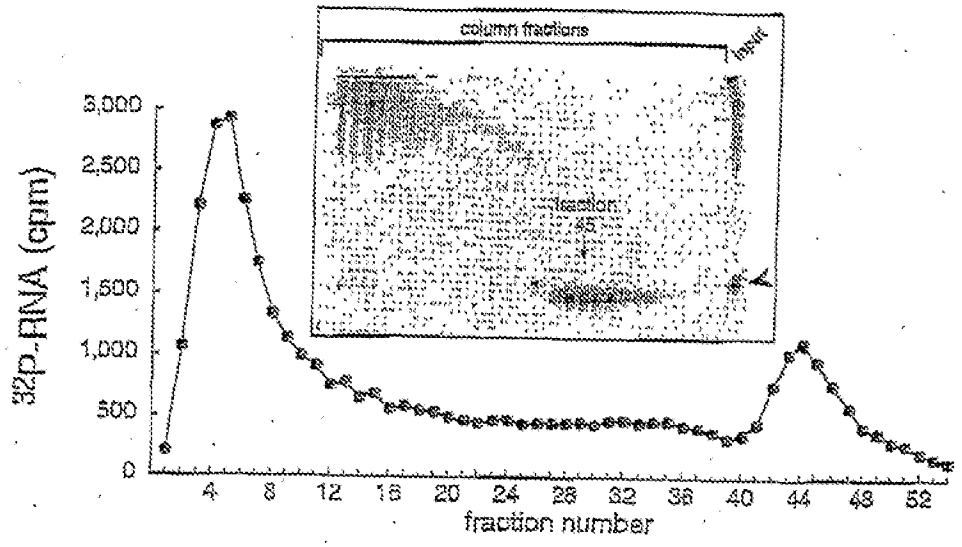


Figure 11

Figure 13A



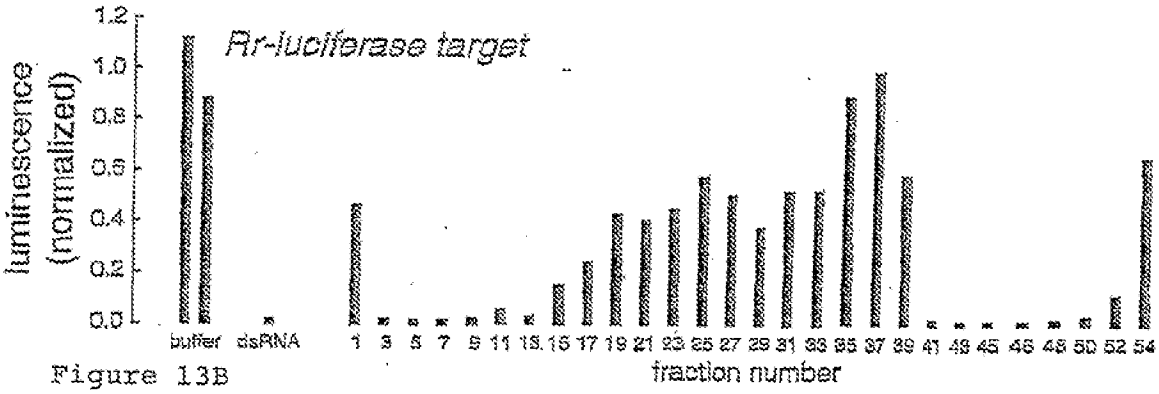


Figure 13B

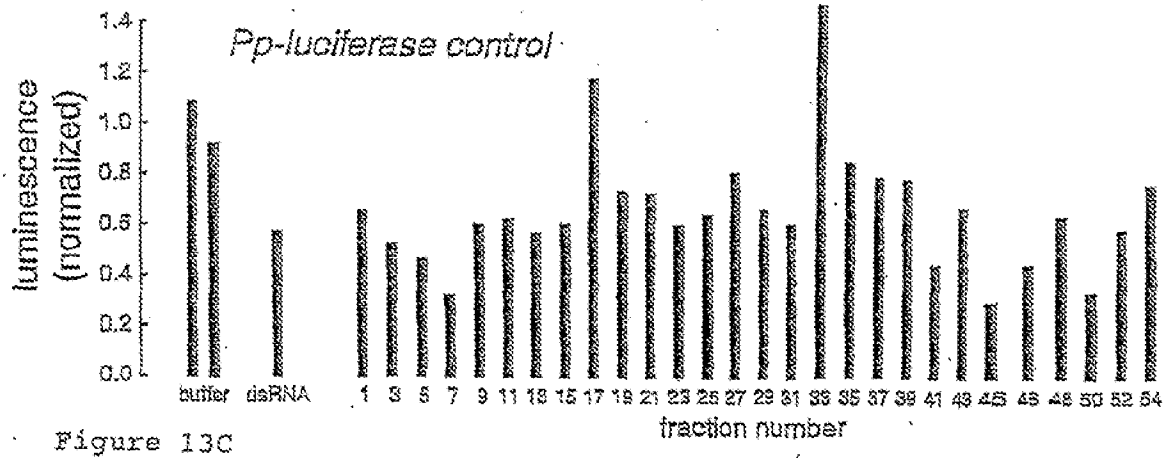


Figure 13C

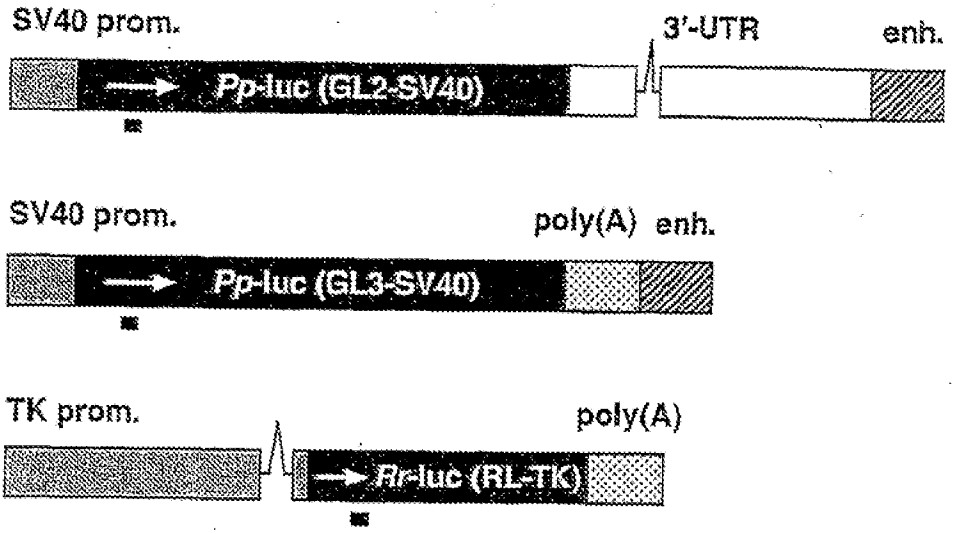


Figure 14A

siRNA duplex	
uGL2	5′ CGUACGCGGAAUACUUCGAUU UUGCAUGCGCCUUAUGAAGCU 5′
GL2	5′ CGUACGCGGAAUACUUCGATT TTGCAUGCGCCUUAUGAAGCU 5′
GL3	5′ CUUACGCUGAGUACUUCGATT TTGAAUGCGACUCAUGAAGCU 5′
invGL2	5′ AGCUUCAUAAGGCGCAUGCTT TTUCGAAGUAUUCCGCGUACG 5′
RL	5′ AAACAUGCAGAAAAUGCUGTT TTUUUGUACGUCUUUUACGAC 5′

Figure 14B

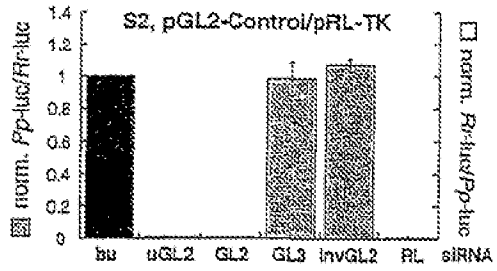


Figure 15A

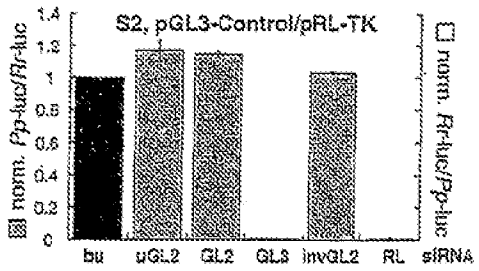


Figure 15B

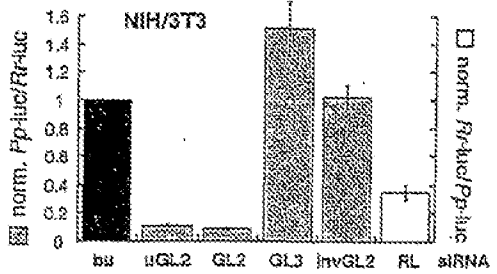


Figure 15C

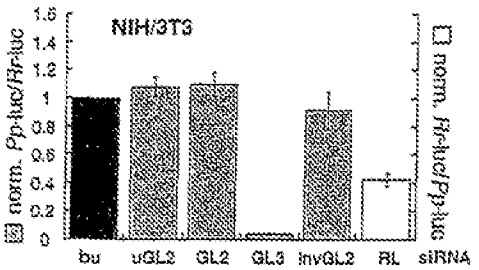


Figure 15D

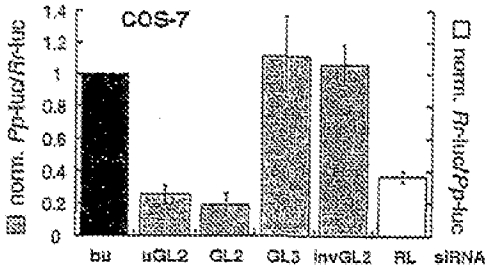


Figure 15E

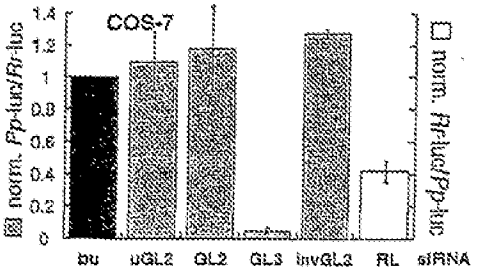


Figure 15F

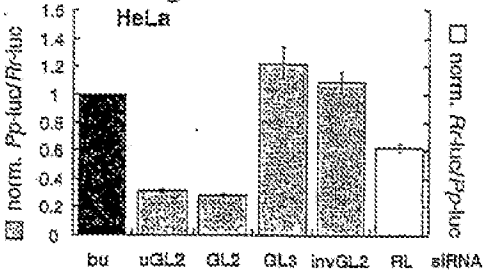


Figure 15G

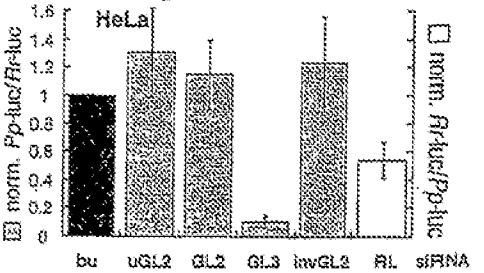


Figure 15H

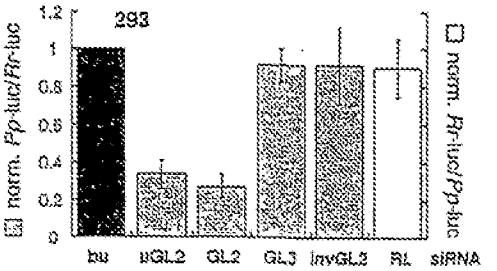


Figure 15I

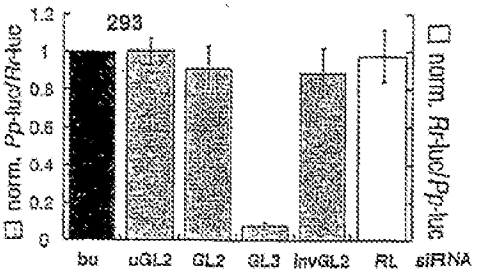


Figure 15J

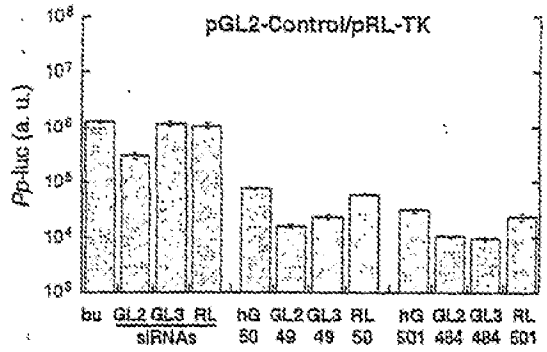


Figure 16A

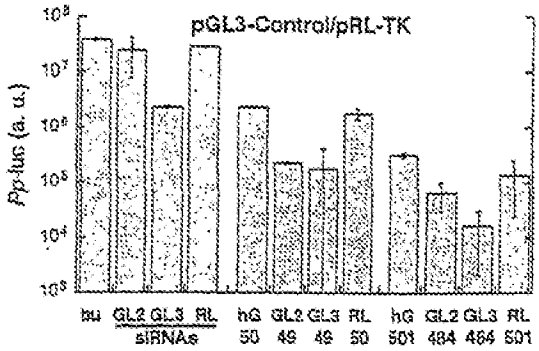


Figure 16B

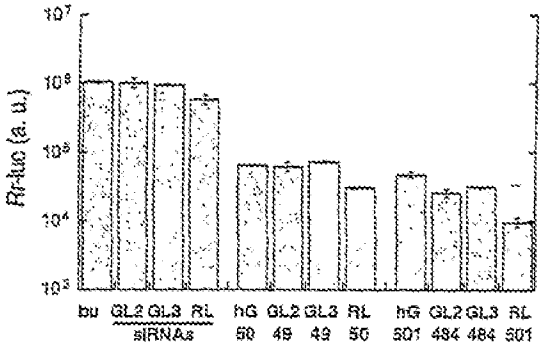


Figure 16C

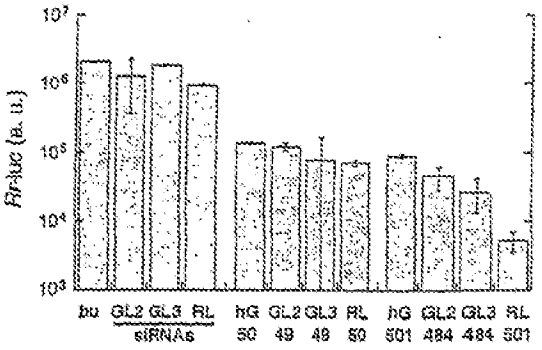


Figure 16D

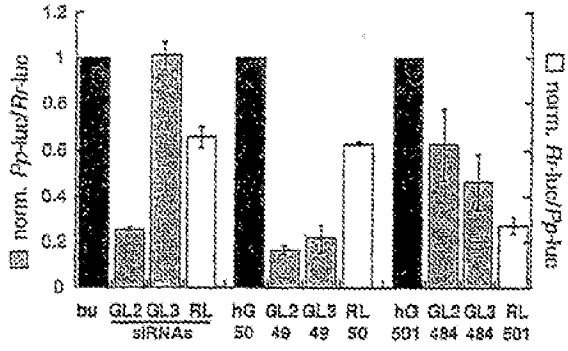


Figure 16E

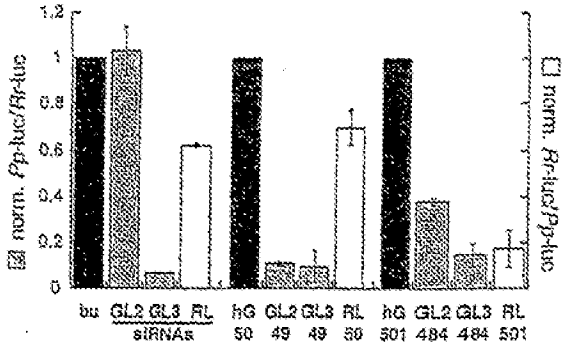


Figure 16F

Electronic Patent Application Fee Transmittal

Application Number:	14462441
Filing Date:	18-Aug-2014
Title of Invention:	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
First Named Inventor/Applicant Name:	Edward Yaworski
Filer:	Joe Chao-Peng Hao/Jose Luna
Attorney Docket Number:	86399-007740US-913296

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:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
		Total in USD ($)		**180**

Electronic Acknowledgement Receipt

EFS ID:	24724833
Application Number:	14462441
International Application Number:	
Confirmation Number:	6562
Title of Invention:	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
First Named Inventor/Applicant Name:	Edward Yaworski
Customer Number:	20350
Filer:	Joe Chao-Peng Hao/Jose Luna
Filer Authorized By:	Joe Chao-Peng Hao
Attorney Docket Number:	86399-007740US-913296
Receipt Date:	26-JAN-2016
Filing Date:	18-AUG-2014
Time Stamp:	14:14:28
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$180
RAM confirmation Number	439
Deposit Account	201430
Authorized User	HAO, JOE C.

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 CFR 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 CFR 1.17 (Patent application and reexamination processing fees)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		US14462441_086399_007740US_IDS_01262016.pdf	208273 07e43e2ef57ba85a756f31e096451d437db02fca	yes	14
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Transmittal Letter		1	4	
	Information Disclosure Statement (IDS) Form (SB08)		5	14	
Warnings:					
Information:					
2	Foreign Reference	JP2002525063.pdf	18565316 6080860b091cf94a21176d5e6953b4a063522cc3	no	83
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5	Foreign Reference	WO0175164.pdf	20526433 4aaed2c4a43bff874fb3ab8460f05bbead659d78	no	70
Warnings:					
Information:					
6	Fee Worksheet (SB06)	fee-info.pdf	30647 d93abbaf243601ddd0982fb0bbfcfc8a88977d8b	no	2
Warnings:					
Information:					

Total Files Size (in bytes):	59629797

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.

I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on January 26, 2016.

KILPATRICK TOWNSEND & STOCKTON LLP

By: /Jose Luna/

PATENT
Attorney Docket No.: 086399-007740US-0913296

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Confirmation No.: 6562
Ed Yaworski et al.	Examiner: Whiteman, Brian A.
Application No.: 14/462,441	Art Unit: 1674
Filed: August 18, 2014	
For: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY	INFORMATION DISCLOSURE STATEMENT
Customer No.: 20350	

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

Commissioner:

The references cited on the attached form PTO/SB/08A&B are being called to the attention of the Examiner. In compliance with the requirements of 37 CFR §1.98(a)(2), copies of reference numbers 110-112 and 132 are submitted herewith. In accordance with 37 CFR §1.98(d), copies of reference numbers 106, 133 and 185 can be found in U.S. Application No. 13/928,309; copies of reference numbers 102-105, 107-109, 126-129, 131, 141, 143-145, 162, 176, 179, 180, 186, 187, 192, 194 and 197-199 can be found in U.S. Application No. 13/253,917; and copies of reference numbers 113-125, 130, 134-140, 142, 146-161, 163-175, 177, 178, 181-184, 188-191, 193, 195, 196 and 200-210 can be found in U.S. Application No. 12/424,367. It is respectfully requested that the cited references be expressly considered during the prosecution of

this application, and the references be made of record therein and appear among the "references cited" on any patent to issue therefrom.

Some of the references cited in this Information Disclosure Statement were cited in Office Actions mailed in connection with commonly-owned U.S. Application Nos. 13/928,309, 13/253,917 and 12/424,367, which share common priority with the subject application. Some of the references cited in this IDS were also cited in Office Actions mailed in connection with commonly-owned U.S. Application Nos. 13/168,543, 11/426,907 and 10/893,121. In addition, Applicant would like to make the Examiner aware of commonly-owned U.S. Application No. 14/494,532. Copies of the Office Actions in these applications are available on PAIR and are believed to be readily accessible to the Examiner.

Commonly-owned patent applications are cited in this Information Disclosure Statement. Copies of any office actions in such patent applications are available on PAIR and are believed to be readily accessible to the Examiner. Thus, although Applicant may submit copies of some such office actions, Applicant does not represent that copies of all, or the most significant, office actions are being supplied. If the Examiner desires copies of all such office actions, the Examiner should contact the undersigned.

As provided for by 37 CFR § 1.97(g) and (h), no inference should be made that the information and references cited are prior art merely because they are in this statement and no representation is being made that a search has been conducted or that this statement encompasses all the possible relevant information.

To satisfy the requirement for a concise explanation of relevance under 37 C.F.R. § 1.98(a)(3)(i) for non-English references cited, please note the following:

Reference 107, JP 03-126211, is not in English. Therefore, we direct the Examiner's attention to the English translation of the abstract which provides a concise explanation of relevance.

Reference 108, JP 05-202085, is not in English. Therefore, we direct the Examiner's attention to the English translation of the abstract which provides a concise explanation of relevance.

Reference 109, JP 06-080560, is not in English. Therefore, we direct the Examiner's attention to the English translation of the abstract which provides a concise explanation of relevance.

Reference 110, JP 2002-525063, is not in English. Therefore, we draw the Examiner's attention to reference 128, WO 00/15820, which is the corresponding English-language document, the abstract of which provides a concise explanation of relevance.

Reference 111, JP 2003-505401, is not in English. Therefore, we draw the Examiner's attention to reference 131, WO 01/05873, which is the corresponding English-language document, the abstract of which provides a concise explanation of relevance.

Reference 112, JP 2007-524349 is not in English. Therefore, we draw the Examiner's attention to reference 137, WO 2004/065546, which is the corresponding English-language document, the abstract of which provides a concise explanation of relevance.

Reference 120, WO 95/18863 A1, is not in English. Therefore, we direct the Examiner's attention to the first page of this reference, which includes an English abstract that provides a concise explanation of relevance.

Reference 122, WO 96/02655, is not in English. Therefore, we direct the Examiner's attention to the first page of this reference, which includes an English abstract that provides a concise explanation of relevance.

The Commissioner is authorized to charge the $180.00 fee set forth in 37 CFR § 1.17(p) to the firm's credit card for consideration of this submission. The Commissioner is also authorized to charge any additional fees due and to credit any overpayment to Deposit Account No. 20-1430.

Respectfully submitted,

/Joe C. Hao/

Joe C. Hao
Registration No. 55,246

KILPATRICK TOWNSEND & STOCKTON LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111-3834
Tel: 925 472-5000
Fax: 415-576-0300
JCH:j3l
Attachments

TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	Docket Number 086399-007740US-0913296

In re Application of: Yaworski *et al.*

Application No.: 14/462,441

Filed: August 18, 2014

For: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

The applicant, PROTIVA BIOTHERAPEUTICS, INC., owner of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of **prior patent No. 9,006,417; prior patent No. 8,492,359; prior patent No. 8,822,668; and prior patent No. 8,236,943** as the term of each said **prior patent** is presently shortened by any terminal disclaimer. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the **prior patent** are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the applicant does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the **prior patent**, "as the term of said **prior patent** is presently shortened by any terminal disclaimer," in the event that said **prior patent** later:

expires for failure to pay a maintenance fee;
is held unenforceable;
is found invalid by a court of competent jurisdiction;
is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
has all claims canceled by a reexamination certificate;
is reissued; or
is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

Check either box 1 or 2 below, if appropriate.

1. ☐ The undersigned is the applicant. If the applicant is an assignee, the undersigned is authorized to act on behalf of the assignee.

I hereby acknowledge that any willful false statements made are punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

2. ☒ The undersigned is an attorney or agent of record. Reg. No. 55246.

/Joe C. Hao/ — Signature
January 27, 2016 — Date

Joe C. Hao

Attorney for Applicant — Title
925 472 5000 — Telephone number

☒ Terminal disclaimer fee under 37 CFR 1.20(d) is included.

Electronic Patent Application Fee Transmittal

Application Number:	14462441
Filing Date:	18-Aug-2014
Title of Invention:	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
First Named Inventor/Applicant Name:	Edward Yaworski
Filer:	Joe Chao-Peng Hao/Judith Cotham
Attorney Docket Number:	86399-007740US-913296

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:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Statutory or Terminal Disclaimer	1814	1	160	160
		Total in USD ($)		**160**

Electronic Acknowledgement Receipt

EFS ID:	24745083
Application Number:	14462441
International Application Number:	
Confirmation Number:	6562
Title of Invention:	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
First Named Inventor/Applicant Name:	Edward Yaworski
Customer Number:	20350
Filer:	Joe Chao-Peng Hao/Judith Cotham
Filer Authorized By:	Joe Chao-Peng Hao
Attorney Docket Number:	86399-007740US-913296
Receipt Date:	27-JAN-2016
Filing Date:	18-AUG-2014
Time Stamp:	19:53:28
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$160
RAM confirmation Number	6379
Deposit Account	201430
Authorized User	HAO, JOE C.

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 CFR 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 CFR 1.17 (Patent application and reexamination processing fees)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		086933_007740_0913296_AMD-TD_012716.pdf	240632 e5b019a0e69696a0657a62755a8cf1ba0c021548	yes	6

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Claims	2	4
Applicant Arguments/Remarks Made in an Amendment	5	5
Terminal Disclaimer Filed	6	6

Warnings:

Information:

2	Fee Worksheet (SB06)	fee-info.pdf	30450 32ade969cb5cb6f1663ba9a16ddceb9b81a903c3	no	2

Warnings:

Information:

Total Files Size (in bytes):	271082

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.

I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on January 27, 2016.

KILPATRICK TOWNSEND & STOCKTON LLP

By: /Judith Cotham/
Judith Cotham

PATENT
Attorney Docket No.: 086399-007740US-0913296

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Yaworski *et al.*

Application No.: 14/462,441

Filed: August 18, 2014

For: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

Customer No.: 20350

Confirmation No. 6562

Examiner: Whiteman, Brian A.

Art Unit: 1674

AMENDMENT

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

Commissioner:

In response to the Office Action mailed October 28, 2015, please enter the following amendments and remarks:

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks begin on page 5 of this paper.

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1-46. (Canceled)

47. (Previously presented) A nucleic acid-lipid particle comprising:

(a) a nucleic acid;

(b) a cationic lipid comprising from 50 mol % to 85 mol % of the total lipid present in the particle;

(c) a non-cationic lipid comprising from 13 mol % to 49.5 mol % of the total lipid present in the particle; and

(d) a conjugated lipid that inhibits aggregation of particles comprising from 0.5 mol % to 2 mol % of the total lipid present in the particle.

48. (Previously presented) The nucleic acid-lipid particle of claim 47, wherein the nucleic acid comprises an interfering RNA, mRNA, an antisense oligonucleotide, a ribozyme, a plasmid, an immunostimulatory oligonucleotide, or mixtures thereof.

49. (Previously presented) The nucleic acid-lipid particle of claim 48, wherein the interfering RNA comprises a small interfering RNA (siRNA), an asymmetrical interfering RNA (aiRNA), a microRNA (miRNA), or mixtures thereof.

50. (Previously presented) The nucleic acid-lipid particle of claim 47, wherein the cationic lipid comprises from 50 mol % to 65 mol % of the total lipid present in the particle.

51. (Previously presented) The nucleic acid-lipid particle of claim 47, wherein the non-cationic lipid comprises a mixture of a phospholipid and cholesterol or a derivative thereof.

52. (Previously presented) The nucleic acid-lipid particle of claim 51, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

53. (Previously presented) The nucleic acid-lipid particle of claim 51, wherein the phospholipid comprises from 3 mol % to 15 mol % of the total lipid present in the particle.

54. (Previously presented) The nucleic acid-lipid particle of claim 51, wherein the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the particle.

55. (Previously presented) The nucleic acid-lipid particle of claim 47, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

56. (Previously presented) The nucleic acid-lipid particle of claim 55, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

57. (Previously presented) The nucleic acid-lipid particle of claim 56, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

58. (Previously presented) The nucleic acid-lipid particle of claim 47, wherein the conjugated lipid that inhibits aggregation of particles comprises from 1 mol % to 2 mol % of the total lipid present in the particle.

59. (Previously presented) The nucleic acid-lipid particle of claim 47, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

60. (Previously presented) A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 47 and a pharmaceutically acceptable carrier.

61. (Previously presented) A method for introducing a nucleic acid into a cell, the method comprising:

contacting the cell with a nucleic acid-lipid particle of claim 47.

62. (Previously presented) A method for the *in vivo* delivery of a nucleic acid, the method comprising:

administering to a mammalian subject a nucleic acid-lipid particle of claim 47.

63. (Previously presented) A method for treating a disease or disorder in a mammalian subject in need thereof, the method comprising:

administering to the mammalian subject a therapeutically effective amount of a nucleic acid-lipid particle of claim 47.

64. (Previously presented) The method of claim 63, wherein the disease or disorder is a viral infection.

65. (Previously presented) The method of claim 63, wherein the disease or disorder is a liver disease or disorder.

66. (Previously presented) The method of claim 63, wherein the disease or disorder is cancer.

REMARKS

I. STATUS OF THE CLAIMS

After entry of this amendment, claims 47-66 are pending and are presented for examination. Claims 1-46 have been canceled without prejudice to future prosecution.

II. NONSTATUTORY DOUBLE PATENTING REJECTIONS

Claims 47-60 were rejected under the judicially created doctrine of obviousness-type double patenting for allegedly being obvious over claims 1-24 of U.S. Patent No. 9,006,417 and claims 1-21 of U.S. Patent No. 8,492,359. Claims 47-66 were rejected under the judicially created doctrine of obviousness-type double patenting for allegedly being obvious over claims 1-23 of U.S. Patent No. 8,822,668. Claims 47-63 and 65 were rejected under the judicially created doctrine of obviousness-type double patenting for allegedly being obvious over claims 1-26 of U.S. Patent No. 8,236,943.

In response, Applicants submit herewith a Terminal Disclaimer to U.S. Patent Nos. 9,006,417, 8,492,359, 8,822,668, and 8,236,943. Accordingly, Applicants respectfully request that the Examiner withdraw the present obviousness-type double patenting rejections.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 925-472-5000.

Respectfully submitted,

/Joe C. Hao/

Joe C. Hao
Reg. No. 55,246

KILPATRICK TOWNSEND & STOCKTON LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111-3834
Tel: 925-472-5000
Fax: 415-576-0300

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/462,441	Filing Date 08/18/2014	☐ To be Mailed

ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT **01/27/2016**

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 20	Minus	** 20	= 0	X $80 =	0
Independent (37 CFR 1.16(h))	* 1	Minus	***3	= 0	X $420 =	0
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	0

AMENDMENT

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))						
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
					TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
/NICOLLE L. SCRIVNER/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
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NOTICE OF ALLOWANCE AND FEE(S) DUE

20350 7590 02/23/2016

KILPATRICK TOWNSEND & STOCKTON LLP
TWO EMBARCADERO CENTER
EIGHTH FLOOR
SAN FRANCISCO, CA 94111-3834

EXAMINER	
WHITEMAN, BRIAN A	
ART UNIT	PAPER NUMBER
1674	

DATE MAILED: 02/23/2016

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/462,441	08/18/2014	Edward Yaworski	86399-007740US-913296	6562

TITLE OF INVENTION: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	05/23/2016

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)

20350 7590 02/23/2016
KILPATRICK TOWNSEND & STOCKTON LLP
TWO EMBARCADERO CENTER
EIGHTH FLOOR
SAN FRANCISCO, CA 94111-3834

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.
14/462,441	08/18/2014	Edward Yaworski	86399-007740US-913296	6562

TITLE OF INVENTION: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	05/23/2016

EXAMINER	ART UNIT	CLASS-SUBCLASS
WHITEMAN, BRIAN A	1674	536-024500

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 — 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 _____ Date _____

Typed or printed name _____ Registration No. _____

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/462,441	08/18/2014	Edward Yaworski	86399-007740US-913296	6562

20350 7590 02/23/2016

KILPATRICK TOWNSEND & STOCKTON LLP
TWO EMBARCADERO CENTER
EIGHTH FLOOR
SAN FRANCISCO, CA 94111-3834

EXAMINER	
WHITEMAN, BRIAN A	
ART UNIT	PAPER NUMBER
1674	

DATE MAILED: 02/23/2016

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. 14/462,441	Applicant(s) YAWORSKI ET AL.	
	Examiner BRIAN WHITEMAN	Art Unit 1674	AIA (First Inventor to File) Status No

-- 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 amendment filed on 1/27/16*.
   ☐ 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 *47-66*. 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. _____ .
   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 a reply 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 _____
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 _____.

/Brian Whiteman/
Primary Examiner, Art Unit 1674

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14462441	YAWORSKI ET AL.
	Examiner	**Art Unit**
	BRIAN WHITEMAN	1674

CPC						
Symbol					**Type**	**Version**
A61K		9	/	1272	F	2013-01-01
A61K		9	/	1271	I	2013-01-01
A61K		48	/	0025	I	2013-01-01
C07H		21	/	00	I	2013-01-01
C07J		9	/	00	I	2013-01-01
C12N		15	/	111	I	2013-01-01
C12N		2310	/	14	A	2013-01-01
C12N		2320	/	32	A	2013-01-01
C12N		15	/	113	I	2013-01-01
			/			
			/			
			/			
			/			
			/			
			/			

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

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	20	
/BRIAN WHITEMAN/ Primary Examiner.Art Unit 1674	2/10/16	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	47	NONE

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14462441	YAWORSKI ET AL.
	Examiner	**Art Unit**
	BRIAN WHITEMAN	1674

US ORIGINAL CLASSIFICATION		INTERNATIONAL CLASSIFICATION										
CLASS	SUBCLASS	CLAIMED					NON-CLAIMED					
		C	1	2	N	15 / 11 (2006.01.01)						
CROSS REFERENCE(S)												
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)											

NONE		**Total Claims Allowed:**	
(Assistant Examiner)	(Date)	20	
/BRIAN WHITEMAN/ Primary Examiner.Art Unit 1674	2/10/16	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	47	NONE

Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14462441	YAWORSKI ET AL.
	Examiner	**Art Unit**
	BRIAN WHITEMAN	1674

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

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original

NONE (Assistant Examiner)	(Date)	**Total Claims Allowed:** 20	
/BRIAN WHITEMAN/ Primary Examiner.Art Unit 1674 (Primary Examiner)	2/10/16 (Date)	O.G. Print Claim(s) 47	O.G. Print Figure NONE

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14462441	YAWORSKI ET AL.
	Examiner	Art Unit
	BRIAN WHITEMAN	1674

CPC- SEARCHED		
Symbol	Date	Examiner
C12N 15/113, 2310/14	2/9/2016	BW
A61K 9/1271	2/9/2016	BW

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Updated Search	2/9/16	BW

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner
C12N	15/113, 2310/14	2/9/2016	BW
A61K	9/1271	2/9/2016	BW

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BIB DATA SHEET

CONFIRMATION NO. 6562

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
14/462,441	08/18/2014	536	1674	86399-007740US-913296

APPLICANTS

Protiva Biotherapeutics, Inc., Burnaby, CANADA

INVENTORS

Edward Yaworski, Maple Ridge, CANADA;
Kieu Lam, Surrey, CANADA;
Lloyd Jeffs, Delta, CANADA;
Lorne Palmer, Vancouver, CANADA;
Ian MacLachlan, Mission, CANADA;

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

This application is a CON of 13/928,309 06/26/2013 PAT 8822668
which is a CON of 13/253,917 10/05/2011 PAT 8492359
which is a CON of 12/424,367 04/15/2009 PAT 8058069
which claims benefit of 61/045,228 04/15/2008

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

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

08/20/2014

Foreign Priority claimed ☐ Yes ☑ No 35 USC 119(a-d) conditions met ☐ Yes ☐ No Verified and Acknowledged /BRIAN A WHITEMAN/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		CANADA	24	20	1

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TITLE

NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	14/462,441
	Filing Date	August 18, 2014
	First Named Inventor	Ed Yaworski
	Art Unit	1674
	Examiner Name	Whiteman, Brian A.
Sheet 1 of 10	**Attorney Docket Number**	086399-007740US-0913296

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /BW/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	14/462,441
	Filing Date	August 18, 2014
	First Named Inventor	Ed Yaworski
	Art Unit	1674
	Examiner Name	Whiteman, Brian A.
Sheet 8 of 10	**Attorney Docket Number**	086399-007740US-0913296

NON-PATENT LITERATURE DOCUMENTS			
Examiner 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.	T[5]
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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /BW/

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	Filing Date	August 18, 2014
	First Named Inventor	Ed Yaworski
	Art Unit	1674
	Examiner Name	Whiteman, Brian A.
Sheet 9 of 10	**Attorney Docket Number**	086399-007740US-0913296

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /BW/

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	14/462,441
	Filing Date	August 18, 2014
	First Named Inventor	Ed Yaworski
	Art Unit	1674
	Examiner Name	Whiteman, Brian A.
Sheet 10 of 10	**Attorney Docket Number**	086399-007740US-0913296

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	209.	WOODLE, M.C., et al., "Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes," Biochimica et Biophysica Acta, 1992, Vol. 1105, pp. 193-200.	☐
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EXAMINER SIGNATURE			
Examiner Signature	/Brian Whiteman/	Date Considered	02/09/2016
*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.			

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Application Number	Application/Control No.	Applicant(s)/Patent under Reexamination	
	14/462,441	YAWORSKI ET AL.	

Document Code - DISQ	Internal Document – DO NOT MAIL

TERMINAL DISCLAIMER	☒ APPROVED	☐ DISAPPROVED
Date Filed : 27 JAN 2016	This patent is subject to a Terminal Disclaimer	

Approved/Disapproved by:
SHANETTE BROWN

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(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/462,441	08/18/2014	Edward Yaworski	86399-007740US-913296	6562

TITLE OF INVENTION: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	05/23/2016

EXAMINER	ART UNIT	CLASS-SUBCLASS
WHITEMAN, BRIAN A	1674	536-024500

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

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(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
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(A) NAME OF ASSIGNEE: PROTIVA BIOTHERAPEUTICS, INC.

(B) RESIDENCE: (CITY and STATE OR COUNTRY): Burnaby, BC Canada

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

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- ☐ Applicant asserting small entity status. See 37 CFR 1.27
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Authorized Signature /Joe C. Hao/ Date May 17, 2016

Typed or printed name Joe C. Hao Registration No. 55246

Electronic Patent Application Fee Transmittal

Application Number:	14462441
Filing Date:	18-Aug-2014
Title of Invention:	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
First Named Inventor/Applicant Name:	Edward Yaworski
Filer:	Joe Chao-Peng Hao/Judith Cotham
Attorney Docket Number:	86399-007740US-913296

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	960	960

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**960**

Electronic Acknowledgement Receipt

EFS ID:	25807329
Application Number:	14462441
International Application Number:	
Confirmation Number:	6562
Title of Invention:	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
First Named Inventor/Applicant Name:	Edward Yaworski
Customer Number:	20350
Filer:	Joe Chao-Peng Hao/Judith Cotham
Filer Authorized By:	Joe Chao-Peng Hao
Attorney Docket Number:	86399-007740US-913296
Receipt Date:	17-MAY-2016
Filing Date:	18-AUG-2014
Time Stamp:	20:44:33
Application Type:	Utility under 35 USC 111(a)

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New Applications Under 35 U.S.C. 111

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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.

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/462,441	06/14/2016	9364435	86399-007740US-913296	6562

20350 7590 05/25/2016

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Atlanta, GA 30309

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):

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