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(12) United States Patent

Papadopoulos et al.

(54) MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES

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- (*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 492 days.

This patent is subject to a terminal disclaimer.

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Related U.S. Application Data

- (60) Continuation of application No. 11/016,097, filed on Dec. 17, 2004, now Pat. No. 7,374,757, which is a division of application No. 10/009,852, filed as application No. PCT/US00/14142 on May 23, 2000, now Pat. No. 7,070,959.
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- (52) **U.S. Cl.** **424/134.1**; 424/192.1; 514/1.1; 514/13.3; 514/21.2; 530/350
- (58) **Field of Classification Search** None See application file for complete search history.

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(10) Patent No.: US 8,029,791 B2 (45) Date of Patent: *Oct. 4, 2011

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(57) ABSTRACT

Modified chimeric polypeptides with improved pharmacokinetics are disclosed. Specifically, modified chimeric Flt1 receptor polypeptides that have been modified in such a way as to improve their pharmacokinetic profile are disclosed. Also disclosed are methods of making and using the modified polypeptides including but not limited to using the modified polypeptides to decrease or inhibit plasma leakage and/or vascular permeability in a mammal.

9 Claims, 55 Drawing Sheets



······∆······ acetylated Flt-1(1-3)-Fc ----O---- unmodified Flt-1(1-3)-Fc





Sheet 3 of 55





Fig.6B.









Fig.10A.

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### Fig.10B.

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Glu	Ile	Gly	Leu	Leu	Thr	Cys	Glu	Ala	Thr	Val	Asn	Gly	His	Leu	Tyr	Lys	Thr	Asn	Tyr>
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GAG	TGT	GTA	GCT	GTT	TGG	TTA	TGT	TAG	TAT	CTA	CAG	GTT	TAT	TCG	TGT	GGT	GCG	GGT	CAG
Leu	Thr	His	Arg	Gln	Thr	Asn	Thr	Ile	Ile	Asp	Val	Gln	Ile	Ser	Thr	Pro	Arg	Pro	Val>
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Lys	Leu	Leu	Arg	Gly	His	Thr	Leu	Val	Leu	Asn	Cys	Thr	Ala	Thr	Thr	Pro	Leu	Asn	Thr>
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TCT	CAA	GTT	TAC	TGG	ACC	TCA	ATG	GGA	CTA	CTT	TTT	TTA	TTC	TCT	CGA	AGG	CAT	TCC	GCT
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# Fig.10C.

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Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp>
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CCG	CAC	CIC	CAC	GTA	TTA	CGG	TTC	TGT	TTC	GGC	GCC	CTC	CTC	GTC	ATG	TTG	TCG	TGC	ATG
Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr>
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GCA	CAC	CAG	TCG	CAG	GAG	TGG	CAG	GAC	GTG	GTC	CTG	ACC	GAC	TTA	CCG	TTC	CIC	ATG	TTC
Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys>
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ACG	TTC	CAG	AGG	TTG	TTT	CGG	GAG	$\mathbf{GGT}$	CGG	GGG	TAG	CTC	TTT	TĠG	TAG	AGG	TTT	CGG	TTT
Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys>
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CCC	GTC	GGG	GCT	CTT	GGT	GTC	CAC	ATG	TGG	GAC	GGG	GGT	AGG	GCC	CTA	CTC	GAC	TGG	TTC
Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Leu	Thr	Lys>
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Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr	Pro	Ser	Asp	Ile	Ala	Val	Glu>
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Trp	Glu	Ser	Asn	Gly	Gln	Pro	Glu	Asn	Asn	Tyr	Lys	Thr	Thr	Pro	Pro	Val	Leu	Asp	Ser>
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CTG	CCG	AGG	AAG	AAG	GAG	ATG	TCG	TTC	GAG	TGG	CAC	CTG	TTC	TCG	TCC	ACC	GTC	GTC	CCC

Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly>

### Fig.10D.

1630 1640 1650 1660 1670 1680 * * * * * * * * * * * * AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser> 1690 1700

* * * * CTC TCC CTG TCT CCG GGT AAA TGA GAG AGG GAC AGA GGC CCA TTT ACT Leu Ser Leu Ser Pro Gly Lys ***>





### Fig.13A.

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ATG	GTC	AGC	TAC	TGG	GAC	ACC	GGG	GTC	CTG	CTG	TGC	GCG	CTG	CTC	AGC	TGT	CTG	CTT	CTC
TAC	CAG	TCG	ATG	ACC	CTG	TGG	CCC	CAG	GAC	GAC	ACG	CGC	GAC	GAG	TCG	ACA	GAC	GAA	GAG
Met	Val	Ser	Tyr	Trp	Asp	Thr	Gly	Val	Leu	Leu	Cys	Ala	Leu	Leu	Ser	Cys	Leu	Leu	Leu>
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mon	COM	101	1001	104	GGI	1CA		114	mmm	GAT	CCT	GAA	CIG	AGT	TTA	-	000	ACC	
161	CCT	AGA	TCA	AGT	CCA	AGT	TTT	AAT	1.1.1.1	CTA	GGA	CTT	GAC	TCA	AAT	J.I.I.	CCG	فاقالا	GIC
unr	GIÝ	ser	Ser	Ser	GTÄ	Ser	Lys	Leu	LYS	Asp	Pro	GIU	Leu	Ser	Leu	Lys	GIY	Inr	GIN>
		13	30		3	L40			150			16	50		. 1	.70			180
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CAC	ATC	ATG	CAA	GCA	GGC	CAG	ACA	CTG	CAT	CTC	CAA	TGC	AGG	GGG	gaa	GCA	GCC	CAT	AAA
GTG	TAG	TAC	GTT	CGT	CCG	GTC	$\mathbf{T}\mathbf{G}\mathbf{T}$	GAC	GTA	GAG	GTT	ACG	TCC	CCC	$\mathbf{CTT}$	CGT	CGG	GTA	TTT
His	Ile	Met	Gln	Ala	Gly	Gln	Thr	Leu	His	Leu	Gln	Cys	Arg	Gly	Glu	Ala	Ala	His	Lys>
		19	90		:	200			210			23	20		:	230			240
	*		*	*		*		*	*		*		*	*		*		*	*
TGG	TOT	TTC:	COT	GAN	አጥር	GING	AGT	DAA	GAA	AGC	GPP	200	CINC	200	ልሞል	እርጥ	222	നവ	CCC
ACC	PCP	220	CCI	COM	mac	CNC	101	mmo	Cam	mog	COM	moo	CAC	mog	mam	11/32	mmm	NGA	000
m	Cor	Lou	Dro	C11	Mat	17-1	500	Tue	C1.	Cox	~1	200	Tan	109		TGR.	T	Cort	2105
тр	Ger	nen	FIU	GIU	nec	vai	Set	uys	Gin	Ser	Gru	мrg	ಗಿರಗ	Ser	тте	1111	цуз	Ser	ALC/
		~							0.00			~	~ ~						200
		2:	50			260			270			2	80			290			300
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1GT	GGA	AGA	AAT	GGC	AAA	CAA	TIC	TGC	AGT	ACT	TTA	ACC	TIG	AAC	ACA	GCT	CAA	GCA	AAC
ACA	CCT	TCT	TTA	CCG	TTT	GTT	AAG	ACG	TCA	TGA	AAT	IGG	AAC	TIG	TGT	CGA	GTT	CGT	TIG
Cys	Gly	Arg	Asn	Gly	Lys	Gln	Phe	Cys	Ser	Thr	Leu	Thr	Leu	Asn	Thr	Ala	Gln	Ala	Asn>
		3:	10		:	320			330			3-	40		:	350			360
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CAC	ACT	GGC	TTC	TAC	AGC	TGC	AAA	TAT	CTA	GCT	GTA	CCT	ACT	TCA	AAG	AAG	AAG	GAA	ACA
GTG	TGA	CCG	AAG	ATG	TCG	ACG	TTT	ATA	GAT	CGA	CAT	GGA	TGA	AGT	TTC	TTC	TIC	CTT	TGT
His	Thr	Gly	Phe	Tyr	Ser	Cys	Lys	Tyr	Leu	Ala	Val	Pro	Thr	Ser	Lvs	Lvs	Lvs	Glu	Thr>
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		3'	70		:	380			390			4	00			410			420
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GAA	TCT	GCA	አጥር	ጥልጥ	ልጥል	<b>T</b> ALLA	እጥጥ	AGT	ርጉም	202	GGT	202	CCT	- TPTC	ርሞአ	242	ATT	ጥልሮ	AGT
CTT	AGA	COT	TAG	2 ጥ እ	ጥልጥ	222	ማ እ	772	CUL	in the second	CCA	ill and in	GGI	220	നമന	Culo	TAC	ביודים	TTC A
G1.	Ser	11a	TIO	There	TIO	Dha	T10	Cor	Acn	Thr	Clu	200	Dro	Dha	Unl	Glu	Mot	m. m	Cors
U.L.u	OCL	mu	41 Ç	+ y r	449	rne	7.7¢	061	hap	TIT	Gtà	πy	ETO	FUG	var	Gru	1100	TÄT	001-
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GAA	ATC	CCC	GAA	ATT	ATA	CAC	ATG	ACT	GAA	GGA	AGG	GAG	CTC	GTC	ATT	CCC	TGC	CGG	GTT
CIT	TAG	GGG	CTT	TAA	TAT	GTG	TAC	TGA	CTT	CCT	TCC	CTC	GAG	CAG	TAA	GGG	ACG	GCC	CAA
Glu	Ile	Pro	Glu	Ile	Ile	His	Met	Thr	Glu	Gly	Arg	Glu	Leu	Val	Ile	Pro	Cys	Arg	Val>
		4	90			500			510			5	20			530			540
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ACG	TCA	CCT	AAC	ATC	ACT	GTT	ACT	TTA	AAA	AAG	TTT	CCA	CTT	GAC	ACT	TIG	ATC	CCT	GAT
TGC	AGT	GGA	TTG	TAG	TGA	CAA	TGA	AAT	TTT	TTC	ААА	GGT	GAA	CTG	TGA	AAC	TAG	GGA	CTA
Thr	Ser	Pro	Asn	Ile	Thr	Val	Thr	Leu	Lys	Lys	Phe	Pro	Leu	Asp	Thr	Leu	Ile	Pro	Asp>

# Fig.13B.

	550 560					570			58	80		5	90			600			
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GGA	ААА	CGC	ATA	ATC	TGG	GAC	AGT	AGA	aag	GGC	TTC	ATC	ATA	TCA	таа	GCA	ACG	TAC	ааа
CCT	TTT	GCG	TAT	TAG	ACC	CIG	TCA	TCT	TTC	CCG	AAG	TAG	TAT	AGT	ATT	CGT	TGC	ATG	TIT
GIY	Lys	Arg	Ile	Ile	Trp	Asp	Ser	Arg	Lys	Gly	Phe	Ile	Ile	Ser	Asn	Ala	Thr	Tyr	Lys>
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GAA	ልጥል	000	ሮጥጥ	CINC	200	ጥርጥ	GDD	602	202	200	ልአጥ	CCC	CAT	002	ጥልጥ	220	202	244	ጥልጥ
CTT	TAT	CCC	GAA	GAC	TGG	ACA	Cm	CGT	TOT	CAG	ጥጥአ	CCC	GTA	AAC	አሞል	mmC.	TCTP	mg	АТА
Glu	Ile	Glv	Leu	Leu	Thr	Cvs	Glu	Ala	Thr	Val	Asn	Glv	His	Leu	Tvr	Lvs	Thr	Asn	Tvr>
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CTC	ACA	CAT	CGA	CAA	ACC	AAT	ACA	ATC	ATA	GAT	GTC	CAA	ATA	AGC	ACA	CCA	CGC	CCA	GTC
GAG	TGT	GTA	GCT	$\operatorname{GTT}$	TGG	TTA	$\mathbf{T}\mathbf{G}\mathbf{T}$	TAG	TAT	CTA	CAG	GTT	TAT	TCG	TGT	$\mathbf{GGT}$	GCG	GGT	CAG
Leu	Thr	His	Arg	Gln	Thr	Asn	Thr	Ile	Ile	Asp	Val	Gln	Ile	Ser	Thr	Pro	Arg	Pro	Val>
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TTT	እአጥ	GAN	- TUCH TUCH	000	CTIN	MC1 MC1	CII	CNG	GNG	17/172	101	ACI	CGA	MCC	MC1 MC1	CCC	230	1011C	
Lvs	Leu	Leu	Ara	Glv	His	Thr	Leu	Val	Leu	Asn	CVS.	Thr	Ala	Thr	Thr	Pro	Leu	Asp	Thr>
				2							010								
		7	90			800			810			8	20			830			840
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AGA	GTT	CAA	ATG	ACC	IGG	AGT	TAC	CCT	GAT	GAA	ATT	GAC	CAA	AGC	AAT	TCC	CAT	GCC	AAC
TCT	CAA	GTT	TAC	TGG	ACC	TCA	ATG	GGA	CTA	CTI	TAA	CTC	GTT	TCG	TTA	AGG	GTA	CGG	TTG
Arg	Val	Gln	. Met	Thr	Trp	Ser	Tyr	Pro	Asp	Glu	Ile	Asr	Gln	Ser	Asn	Ser	His	Ala	Asn>
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TAT	AAG	ATG	TCA	CAA	GAA	TYPA	TAA	CTC	. 19979T	- TAC		TTTC:	- united		יייייי וייזיירי		GAZ	ATZ	TGA
Ile	Phe	TVI	Ser	Val	Leu	Thr	Ile	Ast	) Lvs	Met	Gln	Asr	Lvs	ASC	LVE	Glv	Lei	1 TV1	Thr>
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TGT	CGI	GTA	AGG	AGI	GGA	CCF	TCA	TIC		TCI	GTI	AA	C ACC	TCF	GT	CA1	' ATI	A TA	T GAT
ACA	. GCA	CAT	TCC	: TCA	CCI	GGJ	' AGI	AAC	; TT	AG7	CAA	TI	g TGC	AG1	CAC	GT	TAT I	r ati	A CTA
Cys	Arg	T Val	. Arg	Ser	Gly	Pro	o Ser	- Phe	e Lys	Ser	: Val	ASI	n Thr	: Ser	Val	His	; I10	e Ty:	r Asp>
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ААА	GCA	GGC		GGC	GAG		: AA#	TC	r TGI	GAC		AC	r cad		A TGO	c ccz		3 TG	C CCA
TTT	CGI	r ccc	GGC	: 000	CTC	GGG	TT	AG2	AACI	CTC	TTT	TG	AGIY	G TG		GG	r GG4		GGT
Lys	Ala	Gly	Pro	Gly	r Glu	Pro	b Lys	s Sei	r Cys	a Asi	> Lys	s Th	r His	s Thi	Cy	s Pro	o Pr	o Cy	s Pro>
		10	030		1	040			1050	)		1	060		:	1070			1080
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Ala	. Pro	- CI	l I.e.	j j en		/ G1-	, 000 Pr/	s Aler	r Va	j ph.	ມ ບາຍ ຄຸ ງ. ວາ	J Ph	e Pri	3 333 3 Pm	1 I.I. D I.M	s Pri	атт о Тм	s Ac	o Thr>
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### Fig.13C.

1140 1130 1090 1100 1110 1120 CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp> 1190 1200 1170 1180 1150 1160 * * * CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys> 1250 1260 1230 1240 1220 1210 CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His> 1320 1300 1310 1270 1280 1290 * Ŕ * CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala> 1370 1380 1360 1340 1350 1330 * * CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr> 1440 1420 1430 1410 1400 1390 * * * * CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys> 1500 1490 1480 1450 1460 1470 * * * * GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn> 1520 1530 1540 1550 1560 1510 * * TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu> 1620 1600 1610 1570 1580 1590 * * ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG TEG CAC CTG TTC TCG TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu>

# Fig.13D.

	1630				16	540		1	.650			166	50		16	570	
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GCT	CTG	CAC	AAC	CAC	TAC	ACG	CAG	AAG	AGC	CTC	TCC	CIG	TCT	CCG	GGT	AAA	TGA
CGA	GAC	GTG	TIG	GTG	ATG	TGC	GTC	TTC	TCG	GAG	AGG	GAC	AGA	GGC	CCA	TTT	ACT
Ala	Leu	His	Asn	His	Tyr	Thr	Gln	Lys	Ser	Leu	Ser	Leu	Ser	Pro	Gly	Lys	***;

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ANG	GIC	AGC	AC	- 1' 			maa		CNC	CIG	CIC	200	CGC	GAC	GAG	TC	3 A	CA	GAC	GAA	GAG
TAC	CAG	100	AIL .	э м - m		-10	mbr	Gly	Us]	Lan	Len	Cve	Ala	ī.eu	Leu	Se	r C	vs	Leu	Leu	Leu>
Met	vai	Ser	τŶΙ	c T	ıp /	чэр	THE	GTÅ	vai	Dea	1004	cys	mu	1000	1000			-			
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ACA	GGA	TCT	AG:	r r		39A 2011	001	mon .	CCI	710	CPM	CTC	mac	አጥር	40m	CT	יתי	'AG	GGG	CTT	TAA
191	CCT CUT	AGA	. 10. 60			~1	Cly	bra.	Dro	Dha	Val	Glu	Met	TVY	Ser	Gl	u I	:le	Pro	Glu	Ile>
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MIA	CAC	AIG				COM	A00	000	CIC	CIC	maa	000	ACC		CA	TG	c ı	AGT	GGA	TTG	TAG
TAT	GIG	TAC	فا: ⊫س	A C	~1.1	CC1	100	Cit	Lou	Val	Tie	Pro	CVS	: AT(	v Va	Th	nr s	Ser	Pro	Asn	Ile>
TTe	HIS	met	: Th	rι	eru	σιγ	ALG	GTU	neu	VQL	110	110	031								
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TGG	GAC	AG.	r ac	JA .	AAG	GGC	110	AIC	MIA					את כ הו כ		ייי ייו	TAL.	ጥልጥ	CCC	GAZ	GAC
ACC	CIG		A 10	TT '	TIC	000	AAG	-1AG	TAT	Con	. IIF	, 71-	 	- Du	σ 11 τ Τ.ν	- C	) 11	TIP	Gli	Lei	i Leu>
Trp	Asp	) Se	r Al	rg .	Lys	GTĀ	Pne	116	TTG	: Ser	. <b>ה</b> סו.	I ATO		L LY		a a.	- 4				
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ACC	IG1	GA	a Go	A.	ACA	GIC	MAI	000		. 110 	גרז ג זית איז			η ηγη	с. д.т. Т. д. Т.	a G	AG	TGT	GT	GC	GTT
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Fig.14B.

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GAA	TGA	TAA	CTG	TTT	TAC	GTC	TTG	TTT	CTG	TTT	CCT	GAA -	ATA 	TGA	ACA	GCA	CAT	ICC	TCA Com
Leu	Thr	Ile	Asp	Lys	Met	Gln	Asn	Lys	Asp	Lys	Gly	Leu	Tyr	Thr	Cys	Arg	var	Arg	ser>
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CCT	GGT	AGT	AAG	$\mathbf{T}\mathbf{T}\mathbf{T}$	AGA	CAA	TIG	TGG	AGT	CAC	GTA	TAT	ATA	CTA	TTT	CGT	CCG	GGC	CCG
Gly	Pro	Ser	Phe	Lys	Ser	Val	Asn	Thr	Ser	Val	His	Ile	Tyr	Asp	Lys	Ala	Gly	Pro	Gly>
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Glu	Pro	TAZE	Ser	Cvs	Asn	INS	Thr	His	Thr	Cvs	Pro	Pro	Cvs	Pro	Ala	Pro	Glu	Leu	Leu>
CIU	110	Lys		Cys	1.05	230				-1-			-2						
		7:	30			740			750			70	50			770			780
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CCC	CCT	GGC	AGT	CAG	AAG	GAG	AAG	GGG	GGT	TTT	GGG	TIC	CTG	TGG	GAG	TAC	TAG	AGG	GCC
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Thr	Pro	Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe>
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ATG		-	TGC	ATG	GCA	CAC	CAG	TCO	CAC	GAG	; TGG	CAG	GAC	GIG	GT	CIX	ACC	GAC	TTA
Tyr	TTG	TCG							-	-			TOIL	ı Wie			\ m++) Lei	1 Asn>
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	* TTG Asn	Ser 9	• Thr 70	Tyr	Arg	Val 980	. Val	. Sei	: Val 99(L Leu)	*	10	000 *	*	; GTI	1 ASP 1010 *	, 11 <u></u>	*	1020 *
GGC	*	Ser 9	• Thr 70 •	Tyr	Arg	Val 980 *	Val	. Ser * : TCC	• Val 99(•	Leu) * CAA		10 2 CTC				1 ASI 1010 * C ATV	C GAG	* 3 AAI	1020 * A ACC
GGC	Asn Asn * AAG	Ser 9 GAG	Thr 70 * TAC	Tyr * AAG TTC	Arg TGC	Val 980 * AAG	GTC	* TCC ACC	99(99(AA(TTX	Leu) * CAA/ GTT:		10 C CTC				lolo * C ATY G TA	C GAG	* G AAI C TT	1020 * A ACC I TGG
GGC CCG Gly	; TTG Asn * : AAG ; TTC ; Lys	9 GAG CTC	Thr 70 * TAC ATC	Tyr AAC TTC	Arg TGC ACG Cys	Val 980 * AAG TTC Lys	GTC CAC Val	* C TCC G AGC L Sei	Ya] 99(C AA(C TT C ASI	Leu CAA GTT: DLys	* A GCC T CGC 5 Ala	10 CTC GAG Let)00 * C CCI G GGI 1 Pro	A GCC F CGC D Ala	: GII : : : : : : : : : : : : : : : : : :	LOIO * CATC GTAC OIL	C GAG G CTC e Gli	* G AAI C TT 1 Ly:	1020 * A ACC F TGG s Thr>
GGC CCG Gly	FTIG Asn Asn AAG TTC Lys	GAG GAG GIU	Thr 70 * TAC ATC	Tyr AAC TTC Lys	Arg TGC ACG Cys	Val 980 * AAG TTC Lys	; GTC ; CAC ; Val	* C TCC G AGC L Sei	r Val 99(C AAC G TTX r Asi	Leu AAN G TT: h Lys	* A GCC T CGC S Ala	10 C CTC G GAC)000 * 5 CCI 5 GG1 1 Pro	A GCC F CGC D Ala	: GII : : : : : : : : : : : : : : : : : :	LO10 * CATC GTAC DIL	C GAG G CTV e Gli	* 3 AAJ 2 TT 1 Ly:	1020 * A ACC T TGG 5 Thr>
GGC CCG Gly	* Asn * Asn * AAG TTC * Lys	GAG GAG GAG Glu 10	Thr 70 * TAC ATC TYT	Tyr * AAG TTC : Lys	Arg TGC ACG Cys	Val 980 * AAG TTC Lys 040	GTC CAC Val	* TCC ACC Set	r Val 99(7 2 AAC 3 TTX r Asi 105(Leu AAA G TT D Lys	* A GCC T CGG S Ala	10 CTC GAG Let 10)000 * CCI GGI 1 Pro	A GCC F CGC D Ala	C CCC G GGC G GGC	1 ASP 1010 * C ATC 3 TAC 5 Il 1070	C GAA G CTV E Gl1	* G AAJ C TT 1 Ly:	1020 * A ACC F TGG s Thr> 1080
GGC CCG Gly	* AAG AAG TTC Lys	GAG GAG CTC Glu 10	Thr 70 * TAC ATC Tyr 30 *	Tyr AAG TTC Lys	Arg TGC ACG Cys	Val 980 * AAG TTC Lys 040	GTC CAC Val	* TCC ACC Sec	r Val 99(7 2 AAC 3 TTX r Asi 105(L Leu AAA G TT: h Lys	A GCC T CGG S Ala	10 C CTC GAC Let 10)000 * GG1 Pro	A GCC F CGC Ala	C CCC G GGC G GGC	1 ASP 1010 * 2 ATC 3 TAC 0 Il 1070 *	C GAG G CTV E Gli	* C TT 1 Ly:	1020 * A ACC I TGG s Thr> 1080 *
GGC CCG Gly ATC	Asn Asn AAG TTC Lys *	GAG GAG CTC Glu 10	Thr 70 TAC TAC TAC TYT 30 *	Tyr AAG TTC Lys	Arg TGC ACG Cys 1 A GGG	Val 980 * AAG TTC Lys 040 * CAG	GTC CAC Val	* C TCC G AGC L Set * C CGJ	r Val 99(7 2 AA0 3 TTX r Asi 105(4 GAi	L Leu AAA G TTT G TTT A Lys A CCA	A GCC F CGG S Als A CAC	10 C CTC G GAC Let 10 G GTC)000 * CCF GGI 1 Pro)60 * G TAC	A GCC F CGC D Ala	C CCC G GGC A Pri	LO10 CATC GTAC DI1 LO70 CCC CCC	C GAG C CIX C CIX C CC	* C TT 1 Ly: * A TO	1020 * A ACC T TGG 5 Thr> 1080 * C CGG 5 GCC

Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg>

Fig.14C.

1090 1100 1110 1120 1130 1140 * * * GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT CCG AAG ATA GGG TCG Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser> 1200 1150 1160 1170 1180 1190 * * * * * * * GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro> 1210 1220 1230 1240 1250 1260 * * * * * * * * * CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser> 1270 1280 1290 1300 1310 1320 * * * * * * * * * AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His> 1330 1350 1340 * * * * *

TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA ATG TGC GTC TTC TCG GAG AGG GAC AGA GGC CCA TTT ACT Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>

Fig.15A.

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TAC	CAG	TCG	ATG	ACC	CTG	TGG	ccc	CAG	GAC	GAC	ACG	CGC	GAC	GAG	TCG	ACA	GAC	GAA	GAG
Met	Val	Ser	Tyr	Trp	Asp	Thr	Gly	Val	Leu	Leu	Cys	Ala	Leu	Leu	Ser	Cys	Leu	Leu	Leu>
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TOT	Glv	Ser	Sar	Ser	Gly	GIV	2	Pro	Dhe	Val	Glu	Met	TU-	Ser	Glu	TIA	Pro	Glu	Tle>
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ATA	CAC	ATG	ACT	GAA	GGA	AGG	GAG	CTC	GTC	ATT	CCC	TGC	CGG	GTT	ACG	TCA	CCT	AAC	ATC
TAT	GTG	TAC	TGA	CTT	CCT	TCC	CTC	GAG	CAG	TAA	GGG	ACG	GCC	CAA	TGC	AGT	GGA	TTG	TAG
Ile	His	Met	Thr	Glu	Gly	Arg	Glu	Leu	Val	Ile	Pro	Cys	Arg	Val	Thr	Ser	Pro	Asn	Ile>
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TGA	CAA	TGA	AAT	TTT	TTC	AAA	GGT	GAA	CIG	TGA	AAC	TAC	GG2	CTA	CCT	TTT	GCG	TAT	TAG
Thr	Val	Thr	Leu	Lys	Lys	Phe	Pro	Leu	Asp	Thr	Leu	Ile	e Pro	o Asp	Gly	Lys	Arg	Ile	Ile>
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166	GAC	AGT	AGA	AAG	GGC	TIC	AIC	ATA		AAI			5 TAU		GAR				CIG
ACC	2010	Com	TCT	110		AAG	TAG	TAT	AG1	. 117A		. 1.S.	- MIN	а Т.Т. 1 Т.Т.Т.		- 1240 - 1716		, GAP	
τıμ	Asp	ser	Arg	гуз	σту	Pne	116	116	e ser	ASI	ALC		L IY.	Luys	GIL	1 776	: Grj	1000	Leu-
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ACC	TGT	GAA	GCA	ACA	GTC	AAT	GGG	CAT	TTO	TAT	AAC	ac	A AA	C TAT	CTC			r CGZ	CAA
TGC	ACA	CTT	CGT	TGI	CAG	TTA	ccc	GTZ	AAC	ATA	TT	TG	r TT	G ATZ	GAC	G TG	r GTZ	A GC	GTT
Thr	Cys	Glu	Ala	Thr	Val	Asn	Gly	His	s Leu	Тут	: Lys	s Th	r As	n Tyj	: Leu	i Thi	: His	s Arg	g Gln>
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ACC	I AA I	ACA	ATC	: ATZ	A GAI	GIC	CAA	ATY	A AGO	AC/	A CCI	A CG	c cc	A GT	C AA	A TT	A CT	T AG	A GGC
TGO	TTA	TGI	TAG	TAT	r CTF	CAC	GTI	TAT	r TCC	IG:	r GG	r GC	GGG	T CA	g TT	r aa'	r ga	A TC	r CCG
Thr	Asn	ı Thr	Ile	e Ile	e Asp	val	. Glr	ı Ile	e Sei	: Thi	r Pro	o Ar	g Pr	o Va	l Ly	s Le	i Le	u Ar	a cià>
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AC	TC	A ATC	GGZ	A CT.	A CT	r TT	r TT	TT A	с тс	T CG	a ag	GCZ	NT T	C GC	T GC	T TA	A CI	ig GI	T TCG
Tr) Sei	Ty:	r Pro	o As	p Gl	u Ly	s As	n Ly	s Ar	g Al	a Se	r Va	il Ai	g Ar	g Ar	g Il	e As	sp Gl	n Ser>

Fig.15B.

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 AAG ATG TAT AAG ATG TCA CAA GAA TGA TGA TAA CTG TTT TAC GTC TTG TTT CTG
 Ass Ser His Ala Ass Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Ass Lys Asp>

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 TCA CCT GGT AGT AAG TTT AGA CAA TTG TGG AGT

 Lys Gly Leu Tyr Thr Cys Arg Val Arg
 Ser Gly Pro Ser Fhe Lys Ser Val Asn Thr Ser>

670680690700710720*********GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC ACACAC GTA TAT ATA CTA TTT CGT CCG GGC CCG CCC GGG TTT AGA ACA CTG TTT TGA GTG TGTVal His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr>

730 740 750 760 770 780 * * * * * * * * * * * TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro>

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 TTC GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG CAC CAC CAC CAC
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Fig.15C

1210 1220 1230 1240 1250 12.60 * * * * * * * CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe>

1270 1280 1290 1300 1310 1320 * * * * * * * CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys>

GGT AAA TGA CCA TTT ACT Gly Lys ***>

Fig.16A.

		1	0			20			30			4	0			50			60
	*		*	*		*		*.	*		*		*	*		*		*	*
ATG	GTC	AGC	TAC	TGG	GAC	ACC	GGG	GTC	CIG	CTG	TGC	GCG	CTG	CTC	AGC	TGT	CTG	CTT	CTC
TAC	CAG	TCG	ATG	ACC	CTG	TGG	CCC	CAG	GAC	GAC	ACG	CGC	GAC	GAG	TCG	ACA	GAC	GAA	GAG
Met	Val	Ser	Tyr	Trp	Asp	Thr	Gly	Val	Leu	Leu	Cys	Ala	Leu	Leu	Ser	Cys	Leu	Leu	Leu>
		7	0			80			90			10	0		1	10			120
	*	,	*	*		*		*	*		*		*	*	-	*		*	*
ACA	GGA	TCT	AGT	TCA	GGT	TCA	ААА	TTA	ААА	GAT	CCT	GAA	CTG	AGT	TTA	AAA	GGC	ACC	CAG
TGT	CCT	AGA	TCA	AGT	CCA	AGT	TTT	AAT	TTT	CTA	GGA	CTT	GAC	TCA	AAT	TTT	CCG	TGG	GTC
Thr	Gly	Ser	Ser	Ser	Gly	Ser	Lys	Leu	Lys	Asp	Pro	Glu	Leu	Ser	Leu	Lys	Gly	Thr	Gln>
		13	30		1	40			150			16	50		1	170			180
	*		*	*		*	_	*	*		*		*	*		*		*	*
CAC	ATC	ATG	CAA	GCA	GGC	CAG	ACA	CIG	CAT	CTC	CAA	TGC	AGG	GGG	GAA	GCA	GCC	CAT	AAA
GIG	TAG	TAC	GTT	CGT	CCG	GTC	TGT	GAC	GTA	GAG	GTT	ACG	TCC	CCC	CTT	CGT	CGG	GTA	TIT
His	Ile	Met	Gln	Ala	Gly	Gln	Thr	Leu	His	Leu	Gln	Cys	Arg	Gly	Glu	Ala	ALa	His	Lys>
		19	9 0		:	200			210			2	20		:	230			240
	*		*	*		*		*	*		*		*	*		*		*	*
TGG	TCT	TTG	CCT	GAA	ATG	GTG	AGT	AAG	GAA	AGC	GAA	AGG	CTG	AGC	ATA	ACT	AAA	TCT	GCC
ACC	AGA	AAC	GGA	CTT	TAC	CAC	TCA	TTC	CTT	TCG	CTT	TCC	GAC	TCG	TAT	TGA	TTT	AGA	CGG
Trp	Ser	Leu	Pro	Glu	Met	Val	Ser	Lys	Glu	Ser	Glu	Arg	Leu	Ser	Ile	Thr	Lys	Ser	Ala>
		-																	
		2	50			260			270			2	80			290			300
	*		*	*		*		*	*		*		*	*		*	~	*	
TGT	GGA	AGA	AAT	GGC	AAA	CAA	TIC	TGC	AGT	ACT	TTA	ACC	TIG	AAC	ACA	GCT	CAA	GUA	AAC
ACA	. cor	TCT	TTA	. CCG	1111	GIT	AAG	ACG	TCA	TGA	AAT	166	AAC	116	TGT	CGA	GIT	CGT	11G
Cys	σтγ	Arg	ASN	GIY	Lys	Gin	Pne	Cys	Ser	Inr	Leu	THE	Leu	ASN	mr	AIA	GTU	ALA	ASh>
		3	10			320			330			3	40			350			360
	*		*	*		*		*	*		*		*	*		*		*	*
CAC	ACT	GGC	TTC	TAC	AGC	TGC	AAA	TAT	CTA	GCT	GTA	CCT	ACI	TCA	AAG	AAG	AAG	GAA	ACA
GTG	TGA	CCG	AAG	ATG	TCG	ACG	TTT	ATA	GAT	CGA	CAT	GGA	TGA	AGT	TTC	TTC	TIC	CTT	TGT
His	Thr	Gly	Phe	Tyr	Ser	Cys	Lys	Tyr	Leu	Ala	Val	Pro	Thr	Ser	Lys	Lys	Lys	Glu	Thr>
		-											~~						400
	*	د	70 *	*		380		*	390		*	4	*	*		410 *		*	420 *
GAA	TCT	GCA	ATC	ም አጥ	אידא	T	አጥጥ	AGT	GAT		GGT	AGA	CCI		GTA	GAG	ATG	TAC	AGT
CTI	AGA	CGT	TAG	ATA	TAT	AAA	ТАА	TCZ	CTA	TGI	CCA	TCT	GGJ	AAG	CAT	CTC	TAC	ATG	TCA
Glu	Ser	Ala	Ile	Tyr	Ile	Phe	Ile	Ser	Asp	Thr	Gly	Arg	Pro) Phe	val	Glu	. Met	: Tyr	Ser>
		4	30			440			450)		4	60			470			480
	*		*	*		*		*	*	•	*		*	*		*		*	*
GAA	ATC	ccc	GAA	ATI	ATA	CAC	ATC	ACT	GAP	GGA	AGC	GAG	CIC	GIC	: ATI	r ccc	: TGC	CGG	GTT
CTI	TAG	GGG	CTI	TAA	TAT	GTG	TAC	TG2	CTI		TCC	CTC	GAC	3 CAG	; TAA	GGG	S ACC	GCC	CAA
GTO	11e	Pro	Glu	i Ile	Ile	His	Met	Thi	Glu	ı Gly	' Arg	r Glu	Lei	ı Val	. Ile	Pro	o Cys	s Arg	vai>
		4	90			500			510)		5	520			530			540
	*	-	*	*		*		*	*	r	*	-	*		¢	*		*	*
ACG	TCA	CCT	AAC	ATC	ACT	GTI	ACT	TT	1 AAZ	A AAC	TT	r ccz	A CT	r gao	ACI	TTY	ATA E	e cer	r gat
TGC	AGT	GGA	TIC	TAG	TGA	CAA	TGA	- 44	r TT	TT	: AA	GG!	r Gaj	A CTO	G TGI	A AA(TA	G GGI	A CTA
Thr	Ser	Pro	Asr	l Ile	Thr	Val	Thr	Le	ı Lys	s Lys	s Phe	e Pro	b Lei	u As <u>r</u>	o Thi	: Lei	l Ile	e Pro	> Asp>

Fig.16B.

		55	D		5	60			570			58	0		5	90			600
	*		*	*		*		*	*		*		*	*		*		*	*
GGA	AAA	CGC	ATA	ATC	TGG	GAC	AGT	AGA	AAG	GGC	TTC	ATC	ATA	TCA	AAT	gca	ACG	TAC	ААА
CUT Clu	THE	GCG Name	TAT	TAG	ACC	CIG	TCA	TCT	TTC	CCG	AAG	TAG	TAT	AGT	TTA	CGT	TGC .	ATG	TTT
GTĀ	uys	Arg	тте	тте	Trp	Asp	Ser	Arg	Lys	GIY	Phe	Ile	Ile	Ser	Asn	Ala	Thr	Tyr	Lys>
		61	.0		6	20			630			64	10		e	50			660
	*		*	*		*		*	*		*		*	*		*		*	*
GAA	ATA	GGG	CTT	CTG	ACC	TGT	GAA	GCA	ACA	GTC	AAT	GGG	CAT	TIG	TAT	AAG	ACA	AAC	TAT
CPT	TAT	CCC	GAA	GAC	TGG	ACA	CTT	CGT	TGT	CAG	TTA	CCC	GTA	AAC	ATA	TTC	TGT	TIG	ATA
Gru	TTe	Gιγ	Leu	Leu	Thr	Cys	Glu	Ala	Thr	Val	Asn	Gly	His	Leu	Tyr	Lys	Thr	Asn	Tyr>
		67	0		e	580			690			70	00		7	710			720
	*		*	*		*		*	*		*		*	*		*		*	*
CIC	ACA	CAT	CGA	CAA	ACC	AAT	ACA	ATC	ATA	GAT	GTC	CAA	ATA	AGC	ACA	CCA	CGC	CCA	GTC
GAG	TGT	GTA	GCT	GTT	TGG	TTA	TGT	TAG	TAT	CTA	CAG	GTT	TAT	TCG	TGT	GGT	GCG	GGT	CAG
Leu	Thr	His	Arg	Gln	Thr	Asn	Thr	Ile	Ile	Asp	Val	Gln	Ile	Ser	Thr	Pro	Arg	Pro	Val>
		73	30		•	740			750			7(50			770			780
	*		*	*		*		*	*		*		*	*		*		*	*
AAA	TTA	CTT	AGA	GGC	CAT	ACT	CTT	GTC	CTC	AAT	TGT	ACT	GCT	ACC	ACT	CCC	TTG	AAC	ACG
TTT	AAT	GAA	TCT	CCG	GTA	TGA	GAA	CAG	GAG	TTA	ACA	TGA	CGA	TGG	TGA	GGG	AAC	TTG	TGC
Lys	Leu	Leu	Arg	Gly.	His	Thr	Leu	Val	Leu	Asn	Cys	Thr	Ala	Thr	Thr	Pro	Leu	Asn	Thr>
		7	90		1	B00			810			8:	20		1	830			840
	*		*	*		*		*	*		*		*	*		*		*	*
AGA	GTT	CAA	ATG	ACC	TGG	AGT	TAC	CCT	GAT	GAA	AAA	AAT	AAG	AAC	GCT	TCC	GTA	AGG	CGA
TCT	CAA	GTT	TAC	TGG	ACC	TCA	ATG	GGA	CTA	CTT	TTT	TTA	TTC	TTG	CGA	AGG	CAT	TCC	GCT
Arg	Val	Gln	Met	Thr	Trp	Ser	Tyr	Pro	Asp	Glu	Lys	Asn	Lys	Asn	Ala	Ser	Val	Arg	Arg>
		8	50			860			870			8	80			890			900
	*		*	*		*		*	*		*		*	*		*		*	*
CGA	ATT	GAC	CAA	AGC	AAT	TCC	CAT	GCC	AAC	ATA	TTC	TAC	AGT	GTT	CTT	ACT	ATT	GAC	AAA
GCT	TAA	CIG	GTT	TCG	TTA	AGG	GTA	CGG	TIG	TAT	AAG	ATG	TCA	CAA	GAA	TGA	TAA	CIG	TIT
Arg	Ile	Asp	Gln	Ser	Asn	Ser	His	Ala	Asn	Ile	Phe	Tyr	Ser	Val	Leu	Thr	Ile	Asp	Lys>
		9	10			920			930			9	40			950			960
	*		*	*		*		*	*		*		*	*		*		*	*
ATG	CAG	AAC	AAA	GAC	ААА	GGA	CTT	TAT	ACT	TGT	CGT	GTA	AGG	AGT	GGA	CCA	TCA	TIC	AAA
TAC	GTC	TIG	TTT	CTG	TTT	CCT	GAA	ATA	TGA	ACA	GCA	CAT	TCC	TCA	CCT	GGT	AGT	AAG	TTT
Met	Gin	Asn	Lys	Asp	Lys	Gly	Leu	Tyr	Thr	Cys	Arg	Val	Arg	Ser	Gly	Pro	Ser	Phe	Lys>
		9	70			980			990			10	00		1	010			1020
	*		*	*		*		*	*		*		*	*		*		*	*
TCT	GTT	AAC	ACC	TCA	GTG	CAT	ATA	TAT	GAT	AAA	GCA	GGC	CCG	GGC	GAG	CCC	ААА	TCI	TGT
AGA	CAA	TTG	TGG	AGT	CAC	GTA	TAT	АТА	CTA	. TTT	CGI	CCG	GGC	CCG	CTC	GGG	TTT	AGA	ACA
Ser	Val	Asn	Thr	Ser	Val	His	Ile	Tyr	Asp	Lys	Ala	Gly	Pro	Gly	Glu	Pro	Lys	Ser	Cys>
		10	30		1	040			1050			10	60		1	.070			1080
	*		*	*		*		*	*		*		*	*		*		*	*
GAC	AAA	ACT	CAC	ACA	TGC	CCA	CCG	TGC	CCA	GCA	CCI	GAA	CIC	CTG	GGG	GGA	CCG	TCZ	GTC
CIG	TTT	TGA	GTG	TGT	ACG	GGT	GGC	ACG	GGI	CGI	GGP	CTI	GAC	GAC	ccc	CC1	GGC	AG	CAG
Asp	гув	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro) Ala	Pro	Glu	Lev	Leu	Gly	' Gly	' Pro	Sei	: Val>

Fig.16C.

1100 1140 1090 1110 1120 1130 * * * * * * TTO OTO TTO COO COA AAA COO AAG GAC ACO CTO ATG ATO TOO CGG ACO COT GAG GTO ACA AAG GAG AAG GGG GGT TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr> 1160 * 1170 1180 * * * * 1150 1190 1200 * * * * TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC ACG CAC CAC CTG CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG Cys Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp> 1240 * * 1210 1220 1230 1250 1260 * * * * * GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr> 1280 1290 1300 * * * * * * 1320 * * 1310 * * 1270 * * * * CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG SCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys> 1330 * 1350 * 1360 1370 1380 1340 * * * * * * * THE ANG GTE TEE AND AND GEE CTE CEA GEE CEE ATE GAG ANA ACE ATE TEE ANA GEE ANA ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys>

1400 1410 1420 1430 * * * * * * * * * 1440 1390 * * * * GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys>

1460 * * 1470 1480 1500 1450 1490 1470 * * * * * * * * AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu>

1520 * 1530 * * 1560 * * 1540 1550 1510 1540 1550 * * * * * TEG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser>

1580 * * 1590 * * 1570 1590 1600 1610 1620 * * * * * * * GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC Asp Gly Ser Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly>

Fig.16D.

1640 1680 1630 1650 1660 1670 * * * * * * * * * * * * AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser> 1690 1700

* * * * CTC TCC CTG TCT CCG GGT AAA TGA GAG AGG GAC AGA GGC CCA TTT ACT Leu Ser Leu Ser Pro Gly Lys ***>





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Fig	.21A.			>EcoR	I_site		
10	20	30	40	50	60	70	80
AAGCTTGGGCTGCA	GGTCGATCGA	CTCTAGAGG	ATCGATCCCC(GGCGAGCTCC	GAATTCGCAAC	CACCATGGT	CAGCTAC
IICEACCOACEI		GAGAICICCI	אטר דאופרוע	-CUSCICICAG	CTTAAGCGTTAG	M V	S Y>
						1	4
							>
					>BspEI_bri	.dge	
90	100	110	120	130	140	150	160
TGGGACACCGGGGT ACCCTCTCGCCCCCA	CCTGCTGTGC	GCGCTGCTC	AGCTGTCTGC	ITCTCACAGG	ATCTAGTTCCG	GAGGTAGAC	CTTTCGT
W D T G V	LLC	A L L	SCL!	L L T G	S S>		GAAAGCA
		FLT1 SS_			>	0	
					5	G> >	
						G R	P F V>
							31 >
170	100	100	200		000		0.40
AGAGATGTACAGIG	AAATCCCCGA	AATTATACA	ZOU	ZIU GGAAGGGAGC	ZZU TCGTCATTCCC	Z30 TGCCGGGTI	ACGTCAC
TCTCTACATGTCAC	TTTAGGGGCT	TTAATATGT	STACTGACTT	CETTECETEG	AGCAGTAAGGG	ACGGCCCAA	TGCAGTG
EMIS	E 1 P F	5 I I H	MTE	GRE	L V I P	CRV	т S> 57
			HFL/T1 D2.				>
250	260	270	280	290	300	310	320
CTAACATCACTGTT	ACTITAAAAA	AGTITICCAC	PIGACACTTT	GATCCCTGAT	GGAAAACGCAI	AATCTGGGA	CAGTAGA
PNITV	TLK	K F P 1	AACIGIGAAA 5 D T L	CTAGGGACTA I P D	G K R I	I W D	S R>
			1007 001 DO				84
			REDIT DZ.		4-918		
330	340	350	360	370	380	390	400
TTCCCGAAGTAGTA	TAGTTTACGI	TGCATGTTI	CTTTATCCCG	AAGACTGGAC	ACTICGTIGIC	AGTTACCCG	TAAACAT
KGFII	SNA	түк	EIG	LLTC	ЕАТ	VNG	H L Y>
			HFLT1 D2				۲۲ <
410 TAAGACAAACTATC	420 TCACACATCO	430 ACAAACCAA	440 FACAATCATA	450 GATGTGGTTC	460 TGAGTCCGTCI	470 XATGGAATI	GAACTAT
ATTCIGTTIGATAG	AGTGTGTAGC	TGTTTGGTT	ATGTTAGTAT	CTACACCAAG	ACTCAGGCAGA	GTACCTTAA	CTIGATA
<u>кт N Y</u>	ь т н F нFLT1	содти D2	T I I	 <lu><lu><lu><lu><lu><lu><lu><lu><lu><lu></lu></lu></lu></lu></lu></lu></lu></lu></lu></lu>			
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					HFLK1	D3	

Fig.21B.

	490			500			51	0		5	20			530			54	0			55C)		56	60
GTIGG	AGAA	AAG	CTT	GTC	TTA	LAAT	TGT	ACA	GCA	AGA	ACI	GAA	CTA	TAA	GTG	GGG.	ATT	GAC	TT	CAZ	1CTC	3GGA	AT7	1CCC	CT
CAACC	TCTI	TTO	GAA	CAG	LAA T	ATT'	ACA	TGI	CGI	TCT	TGA	CTT	GAI	TTA	CAC	CCC	TAA	CIX D	iaa T	GTI	IGAC	1001 7	rAT.	rgg(v v	GA D
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	570			580)		59	0		6	500			610)		62	0			630)		6	40
TTCGA	AGC	ATCA	IGCA	AAT.	GAP	AACI	PIGI		1CCC	SAGA	ICC1				GTC	TGG	GAG	TG/	AGA non	TG/	AG2	AAA'.	1111. DDD	PIG	AG
AAGUI	KF	IAGI I O		I K	Curi T	K I	LACA L V	7 N	JF	S E	ioor) I) K		r C) S	G	5	.мс.	E	M.	K K	K	F	L	S
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NGGAC	a contraction	CIG CIG	D D	2 T CZ	nga V	r F	7. T.	ruu T	p	Этт	K IG	PJ	r CC K	ינטי	ссса т	[, ]	а а	T	s	R	T	P	E	v	
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IGCGT	GGTG	GTG	GAC	GTG	AGC	CAC	GAA	GAC	CCT	GAG	GTC	AAG	TTC	AAC	TGG	TAC	FIG	GAC	CGG	CGI	GGA	'GG'	IGCI	ATA	A'11
ACGCA	CCAC	CAC	CIG	CAC.	ICG	GTG	CTT	CTG	GGA	CTC	CAG	TTC	AAG	TTG	ACC.	ATG	CAC		жc	GCF	.CC1	.'CCA	الحالية.	TAT.	TA:
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GGTGG CCACC V 1	ACGI TGCA D V	GAGC CTCC S	CAC GTG H	GAA CTT E 98	GAC CTC D	CCCI 3GGA P	GAG CTC E 9	GTC: CAG' V F ⁽ 90	AAG' FTC K CΔC	TTCA AAGT F 1 - 10	ACTO TGAO N V A AI	GTA CAI V Y LLOI	CGTG( GCAC( V TYPE	EACO CTG D	GGCC CCGC G	5TG( 2AC( V 	E E E 20	GTC CAC V	CAT CGT7 H	N N	GCC LCGG A	AAG TTC K	ACAA TGTT T> 297 1040
GGTGGI CCACC' V 1	ACGI IGCA D V  97 CGGG	GAGC CTCC S 0 AGGF	CAC GTG H	GAA CTT E 98	GAC CTC D  0 CAZ	CCT 3GGA P 	GAG E E 9 CAC	GTC: CAG' V F( 90 GTA(	AAG' FTCI K CΔC	ITCA AAGI F 1 - 10 TGTG	ACTO TGAO N V A AI 000 GTCZ	GTA CAT V Y LLOI	CGTG GCAC V TYPE 101 TCCT	DACO D D	GGCC CCGC G	5TG( CAC( V 10: CCT(	EAGE E 20 GCA	GTC CAC V	CAT CGT# H	N N LO3C	GCC A A GCT	AAG TTC K	ACAA TGTT T> 297 1040 TGGC
GGTGGI CCACC ⁴ V 1 AGCCGC TCGGCC	ACGI TGCA D V 97 CGGG GCCC	GAGC CTCC S 0 AGGF TCCT	CAC GTG H	GAA CTT E 98 GTA	GAC D D CAF GTT	CCT 3GGA P ACAG	GAG E E 9 CAC	GTC/ CAG' V F( 90 GTA( CATY	AAG' FTC: K CAC CCG'	ITCA AAGI F 1 - 10 TGTG ACAC	ACTO TGAO N V A AI 000 GTC/ CAGI	KGTA ICAI V Y LLOI NGCG	CGTG GCAC V TYPE 101( TCCT AGGA	EACO D D CACO ETG	GGCC G G CGTX GCAC	IO: CCTV	EAG E E 20 GCA	STC CAC V	CGTZ H 1 AGGZ	IN N LO3C	GCC A A GCT CGA	AAG TIC K GAA	ACAA TGTT 297 1040 TGGC
GGTGGI CCACC ⁴ V 1 AGCCG4 TCGGC4 K P	ACGI TGCA D V 97 CGGG GCCC R	GAGC CTCC S 0 AGGZ TCCT E F	CAC GTG H	98 GAA E 98 GTA CAT	GAC D D CAZ GTI	CCCI 3GGA P ACAG NGTC N S	GAG E 9 CAC GTG T	GTC/ CAG' V 90 GTA( CATV Y	AAG ITCI K CAC CCG SGCI R	ITCA AAGI F 1 - 10 IGIG ACAC V	ACTO TGAO N V A AI 000 GTCZ CAGI V	KGTA XCAI V Y LLOI AGCG ICGC S	CGTGC GCACC V TYPE	EAC TG D D CAC ETG T	GGCC G G CGTC GCAC V	IO: CACO V 10: CCTO GGAO L	EAG E 20 GCA GCA H	STC CAC V	CAJ CGTZ H 1 AGGZ PCC 2 I	IDAT N N LO3C NCTC IGAC	GCC A A GCCI CCGA J L	AAG TTC K GAA CTI	ACAA TGTT 297 1040 TGGC ACCG S
STGGI ZACC ⁴ V 1 SCCG4 SCCG4 P	ACGI TGCA D V 97 CGGG GCCC R	GAGC CTCC S 0 AGGZ TCCT E E	CAC GTG H GCA GCA	98 GAA E 98 GTA CAT	GAC CTK D 0 CAZ GTJ	CCCI JGGA P ACAG IGTC N S	GAG E 9 CAC GTG T	GTC CAG V 90 GTA CAT Y	AAG' FTCJ K CAC CCG' 3GCJ R	ITCA AAGI F 1 - 10 IGTG ACAC V 1 -	ACTO TGAO N V A AI 000 GTCZ CAGI V A AI	GTA CAT V Y LLOT AGCG S	CGTGC GCACC V TYPE 1010 TCCTC CAGGAC V L	JACO D D CACO JCACO T	GGCC G G CGTC GCAC V	IO: CACO V 10: CCTV SGAO L	EAG E 20 GCA GCA H	STC CAC V CC2 SC2 SC2 CC2	ICCT CGTZ H 1 AGGZ ICCT Q I	N N LO3C N COTO NGAC	GCC A GCT GCT CCA J L	AAG TTC K GAA CTI , N	ACAF TGT1 T> 297 1040 TGGC ACCG ( G> 32

# Fig.22C.

TCCTC K E CGAGA GCTCT R E	113 ACC	0 ACA0	C	TCC	V V	AGG' S	rig N	TTT K	CGG Α _FCΔ	GAG L	GGT P - A	CGG A	GGG P	TAG	CTC E	TTT K	TGG T	TAG I	AGG	TTI K	YCGG A	TTI K	000 000 G	GTC Q	GG P>
CGAGA GCTCT R E	113 ACC								FCΔ	.C1	- A	AL	т.От		£,	Г	1	Ŧ	2	v	A	A	G	Ŷ	22
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CGAGA SCTCT R E	113 ACC	0 ACAC										<u></u>	× ۲۰	- 1 1 /		~~									
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GAGA GCTCT R E		ACAL	-	114	10 1200	2017	1	150		~~~	116	0	~~~	11'	70	~	1	180			119	0		12	00
RE	166	TGT	CAC	ATG	ACC FIGG	GGA(	CGG	GGG	TAG	GGC	GGA CCT	'ACT	CGA	GACO CTGO	jaa Gtt	GAA CTT	CCA GGI	GG1 CCA	'CAG .GTC	ICC'I IGGA	GAC CTG	CTC GAC	ICC'I IGGA	CCA	CA GT
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AGGCT	TCT	ATCO	CAC	CGA	1CA7	rcg	CCG	TGG	AGT	GGG	AGA	.GCA	ATG	GGC	AGC	CGG	AGA	ACA	ACT	ACA	AGA	CC4	CGC	CTC	:CC
TCCGA	AGA	TAGO	GTC	GCI	GTA	fec.	GGC	ACC	TCA		TCT	'CGT	TAC	CCG'	rcg	GCC	TCT	TGT	TGA	TGI	TCI	GGI	GCC	GAG	GG
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AUGAC V L	D D	AGG S	D D	Aنتا⊃: ج	منی کی ا S	F.	AAG F	GAG T.	ATA V	nce s	TLC K	GAG T.	ידי יד	CAC	CIG	TTC	TCG	TCC	'ACC W	GTC	GTC	200	TTC. M		AA: T
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ICATG	GAG	GCAC	- TAC	GTA	CIC	CG	AGA	CGT	GTT	GGT	GAT	GIG	CGT	CTT	CTC	GGA	GAG	GGA	CAG	AGG	CCC	ATI	TAC	TCG	ЮC
ICATG AGTAC S C	CTC GAG S	GCAC V	M	GTA H	ACTC E	CG: A	AGA L	CGT H	GTT I N	GGT H	GAT Y	GTG T	KGT VQ	CTT K	CTC S	GGA L	GAG S	GGA L	CAG	AGG	CCC G	ATI F	TAC	77CG >	ксс

CCGC GGCG



Fig.24A. 50 60 20 30 40 * * ATG GTC AGC TAC TOG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC TAC CAG TOG ATG ACC CTG TOG CCC CAG GAC GAC ACG CGC GAC GAG TOG ACA GAC GAA GAG M V S Y W D T G V L L C A L L S C L L L> 20> 15 1 5 110 120 70 80 90 100 * * * * * ACA GEA TCT AGT TCC GGA AGT GAT ACC GGT AGA CCT TTC GTA GAG ATG TAC AGT GAA ATC TGT CCT AGA TCA AGG CCT TCA CTA TGG CCA TCT GGA AAG CAT CTC TAC ATG TCA CTT TAG TGSSSG> 21_hFLT1 SIGNAL SEQ_26> SDTGRPFVEMYSE I> 40> _30___hFL/T1 IG DOMAIN 2_ __27_ 170 180 150 160 130 140 * * CCC GAA ATT ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT ACG TCA GOG CTT TAA TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA TGC AGT PEIIHMTEGRELVIPCRVTS> ___hflti ig domain 2___ 55 60> _45_ 41 240 220 230 190 200 210 * * * * * CCT AAC ATC ACT GIT ACT TTA AAA AAG TIT CCA CIT GAC ACT TTG ATC CCT GAT GGA AAA GGA TTG TAG TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA CCT TTT PNITVTLKKFPLDTLIPDGK> 80> 65 ___hfi/f1 ig domain 2__ 75 61 300 280 290 270 250 260 * * * * * CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA GAA ATA GCG TAT TAG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT CTT TAT R I I W D S R K G F I I S N A T Y K E I> ____hFLT1 IG DOMAIN 2_____95_ 100> _85_ 81 350 360 340 310 320 330 * * GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT CTC ACA CCC GAA GAC TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA GAG TGT G L L T C E A T V N G H L Y K T N Y L T> ____hfl/T1 IG DOMAIN 2____ 115 120> _____105_ 101____ 420 370 380 390 400 410 * * * * * CAT CGA CAA ACC AAT ACA ATC ATA GAT GIG GTT CIG AGT CCG TCT CAT GGA ATT GAA CTA GTA GCT GTT TGG TTA TGT TAG TAT CTA CAC CAA GAC TCA GGC AGA GTA CCT TAA CTT GAT H R Q T N T I I D> 121____hFLT1 IG DOMAIN 2__ _129_> VVLSPSHGIEL> 140> hFLK1 IG DOMAIN 3____ 130

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TCT AGA S 141_	GIT CAA V	GGA CCT G	* GAA CTT E	AAG TTC K 145_	CTT GAA L	* CAG V	TTA AAT L h	AAT TTA N FLK1	* IGT ACA C IG	ACA TGT T DOMA	GCA CGT A LN 3	AGA TCT R	* ACT TGA T	GAA CTT E _155_	CTA GAT L	* AAT TTA N	GTG CAC V	999 CCC G	* ATT TAA I> 160>
		49	•0		5	500 *			510 *			52	20		5	530 *			540 *
GAC CTG D 161_	TIC AAG F	AAC TIG N	TGG ACC W	GAA CTT. E 165_	TAC ATG Y	CCT GGA P	TCT AGA S hFI	TOG AGC S K1 I	AAG TTC K G D	CAT GTA H MAIN	CAG GIC Q I 3_	CAT GTA H	AAG TTC K	AAA TTT K _175_	CTT GAA L	GTA CAT V	AAC TIG N	CGA GCT R	GAC CIG D> 180>
		55	50 *		5	560 *			570 *			58	B0 *		!	590 *			600 *
CTA GAT L 181_	AAA TTT K	ACC TGG T	CAG GTC Q	TCT AGA S _185_	GGG CCC G	AGT TCA S	GAG CTC E 1	ATG TAC M DFLKI	AAG TIC K IG	AAA TTT K DOMP	TTT AAA F AIN 3	TIG AAC L	AGC TCG S	ACC TGG T _195_	TTA AAT L	ACT TGA T	ATA TAT I	GAT CTA D	GGT CCA G> _200>
		61	10 *		1	520 *			630 *			6	40 *			650 *			660 *
GTA CAT V 201	ACC TGG T	CGG GCC R	AGT TCA S	GAC CIG D _205_	CAA GIT Q	GGA CCT G	TTG AAC L h	TAC ATG Y FLK1	ACC TGG T IG	TGT ACA C DOMA	GCA CGT A IN 3	GCA CGT A	TCC AGG S	AGT TCA S _215	GGG CCC G	CIG GAC L	ATG TAC M	ACC TGG T	AAG TTC K> _220>
		6	70 *			680 *			690 *			7	00 *			710 *			720 *
AAG TTC K 221	AAC TIG N	AGC TCG S	ACA TGT T	TTT AAA F	GTC CAG V	AGG TCC R MAIN	GIC CAG V 3	CAT GTA H	GAA CIT E	AAG TIC K> 231	GAC CIG	AAA TTTT	ACT TGA	CAC GTG	ACA TGI	ACC	CCA GGT	CCG GGC	TGC ACG
£-2					0.00		·				D 232	ĸ	T	H hFC	T AC1	C A	P	P	C> _240>
		7	30 *			740 *			750 *			7	'60 *			770 *			780 *
CCA GGT P 241	GCA CGT A	GGA P	GAA CTT E	CIC GAG L _245	CTG GAC L	GGG CCC G	GGA CCT G	CCG GGC P h	TCA AGI S FCAC	GIC CAG V 1 A	TTC AAG F	CTC GAG L	TTC AAC F	CCC GGG P _255	CCZ GGI P	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	A CCC r GGG P	C AAG TIC K	GAC CIG D> _260>
		7	90 *			800 *			810	)		٤	320 *			830 *			840 *
ACC TGG T 261	CIC GAG L	ATG TAC M	ATC TAG I	TCC AGG S _265	CGG GCC R	ACC TGG T	CCI GGA P	GAG CTC E h	GTC CAG V FCAC	TGT TGT T	TGC ACC C	CAC	G GTC CAC V	GIG CAC V _275	GAC CTX D	C GTX G CA( V	G AGO C TCO S	CAC G GTC H	CTT E> 280>
		8	50 *			860 *			870	) +		8	380 *			890 *			900 *
GAC CTG D	CCI GGA P	GAG CIC E	GTC CAG V	AAG TTC K	TTC AAC F	CAAC TTC N	TGG ACC W	TAC TAC TA Y	GTC CAC V	G GAC C CTG D C1 A	G G G	GIN GCA V	GAC CCIV E	GTC CAC V 295	GTX GTX H	r aa' a TT N	r GCC A CGC A	C AAC G TTK K	G ACA C TGT T> 300>

Fig		24C.
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	910 *				920 9 *				930 940 * *			94	0	950 *				960 *		
AAG TTC K	CCG GGC P	CGG GCC R	GAG CIC E	GAG CIC E 305	CAG GTC Q	TAC ATG Y	AAC . TTG ' N	AGC TCG S bF	ACG TGC T	TAC ATG Y A	CGT GCA R	GTG CAC V	GTC CAG V	AGC TCG S 315	GTC CAG V	CIC GAG L	ACC TGG T	GIC CAG V	CTG GAC L> 320>	
		97	0		9	B0			990			100	0		10	10		1	.020	
CAC GTG H	CAG GTC Q	GAC CIG D	* TGG ACC W	CTG GAC L	aat tta N	* 222 202 G	AAG TTC K	GAG CTC E	TAC ATG Y	AAG TTC K	TGC ACG C	AAG TTC K	GTC CAG V	TCC AGG S	AAC TIG N	AAA TTT K	GCC CGG A	CTC GAG L	CCA GGT P> 340>	
321_		103	30	_343 <u>_</u>	10	)40		.ب 1	16CDC	.1 A	<b>A</b> rthurston	106	50		1	070		•	1080	
GCC	000	ATC	* GAG	AAA ידידידי	ACC	* ATC TAG	TCC AGG	AAA TTT	* GCC CGG	AAA TTT	GGG CCC	CAG GTC	* CCC GGG	CGA GCT	GAA CTT	* CCA GGT	CAG GTC	GIG CAC	* TAC ATG	
A 341	P	I	E	к _345_	T	I	S	K hI	A FCAC	K LA	G	Q	P	R _355_	E	P	Q	v	¥> _360>	
		10	90 *		1:	LOO *		:	1110			11:	20 *		1	130 *			1140 *	
ACC TGG T	CTG GAC L	CCC GGG P	CCA GGT P	TCC AGG S	CGG GCC R	GAT CTA D	GAG CTC E	CIG GAC L	ACC TGG T	AAG TTC K	AAC TIG N	CAG GTC Q	GTC CAG V	AGC TCG S	CIG GAC L	ACC TGG T	TGC ACG C	CTG GAC L	GTC CAG V>	
361				_365_				h	FCAC	1 A .				_375		100			_380>	
		11	50 *		1	160 *			*			ΤŤ	*		1.	*			*	
AAA TTT K	GGC CCG G	TTC AAG F	TAT ATA Y	CCC GGG P	AGC TCG S	GAC CTG D	ATC TAG I	GCC CGG A	GIG CAC V	GAG CTC E	TGG ACC W	GAG CTC E	AGC TCG S	AAT TTA N	GGG CCC G	CAG GTC Q	CCG GGC P	GAG CIC E	AAC TIG N>	
381			<del></del>	_385				h	FCAC	1 A .				_395					_400>	
		12	10 *		1	220 *			1230 *			12	40 *		1	250 *			1260 *	
AAC TIG N	TAC ATG Y	AAG TTC K	ACC TGG T	ACG TGC T	CCT GGA P	CCC GGG P	GTG CAC V	CTG GAC L	GAC CTC D	TCC AGG S	GAC CIG D	GGC CCG G	TCC AGG S	TTC AAC F	TTC AAG F	CIC GAC L	: TAC HATG Y	AGC TCC S	C AAG G TIC K>	
401				405				n	FCAC	LA				_415						
		12	70 *		1	280 *			1290	) -		13	*		]	.310 *			1320	
CIC GAG L	ACC TGG T	GTG CAC V	GAC CTG D	AAG TIC K	AGC TCG S	AGG TCC R	TGG ACC W	CAG GTC Q	CAG GTC Q	600 CCC G	AAC TTG N	GTC CAG V	TTC AAG F	TCA GAGI S	ACC C	TCC AGC S	GTG GCAC V	NTA ( NAT : M	G CAT C GTA H>	
421				_425				h	FCAC	1 A				_435		_			440>	
		13	30 *		1	340 *			1350 ,	) r		13	860 *		3	L370 *				
GAG CTC	GCI	CIG	CAC	AAC	CAC	TAC	ACG	CAG	AAC	AGC	CIC	: TCC	c cro	TCI	. ccc	GG	r aaf	I TG	A	
	CGF	A GAC	GIG	TTG	GIG	ATG	TGC	GTC	TT	: 100	GAG	AGO	GAC	C AG2	GGC		A TT	r ac	T	













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11D2Flk1D3.FcΔC1(a) & VEGFR1R2-FcΔC1(a)	VEGF/VEGFR1R2-FcAC1(a)	0.98	0.94	0.99		0.97 ± 0.02	
oichiometry of hVEGF165 to F	VEGF/FIt1D2FIk1D3.FcAC1(a)	0.93	0.97	-		$0.96 \pm 0.03$	
Binding St	hVEGF165 (nM)	-	10	50		Average ± StDev	

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Free Flt1D2Flk1D3.FcΔC1(a) (M)

5E-10

0E+00

Fig.29. 1E-09 1E-09 4 8E-10 5E-10 0E+00 (2E-10) Fig.29. Y Intercept = 0.989 nM X Intercept = 0.936 nM Slope = -1.06

> 1E-09 2E-09 2E-09 Total VEGF Added (M)

3E-09

3E-09

Fig.30.











Fig.36.
GRPFVEMYSEIPEIIHMTEGRELVIP <u>C</u> RVTSP <u>N</u> ITVTLKKFPLDTLIPDG
KRIIWDSRKGFIIS <u>N</u> ATYKEIGLLT <u>C</u> EATVNGHLYKTNYLTHRQTNTIID
150
VVLSPSHGIELSVGEKLVL <u>NC</u> TARTELNVGIDFNWEYPSSKHQHKKLVNR
200
DLKTQSGSEMKKFLSTLTIDGVTRSDQGLYTCAASSGLMTKKNSTFVRVH
250
EKGPGDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD
VSHEDPEVKFŇWÝVDGVEVHNAKTKPREEQY <u>N</u> STYRVVSVLTVLHQDWĽŇ
350
GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL
400
TCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS
RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Sheet 51 of 55







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# MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES

This application is a continuation of U.S. patent application Ser. No. 11/016,097, filed Dec. 17, 2004, now U.S. Pat. No. 7,374,757,which is a divisional of U.S. patent application Ser. No. 10/009,852, filed Dec. 6, 2001, now U.S. Pat. No. 7,070,959, which is a national stage application of International Application No. PCT/US00/14142, filed May 23, 2000, ¹⁰ which claims priority of U.S. Provisional Application No. 60/138,133, filed Jun. 8, 1999. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

# INTRODUCTION

The field of this invention is modified polypeptides with improved pharmacokinetics. Specifically, the field of this invention relates to Flt1 receptor polypeptides that have been 20 modified in such a way as to improve their pharmacokinetic profile. The field of this invention also relates to methods of making and using the modified polypeptides including but not limited to using the modified polypeptides to decrease or inhibit plasma leakage and/or vascular permeability in a 25 mammal.

## BACKGROUND

The ability of polypeptide ligands to bind to cells and 30 thereby elicit a phenotypic response such as cell growth, survival, cell product secretion, or differentiation is often mediated through transmembrane receptors on the cells. The extracellular domain of such receptors (i.e. that portion of the receptor that is displayed on the surface of the cell) is gener- 35 ally the most distinctive portion of the molecule, as it provides the protein with its ligand binding characteristic. Binding of a ligand to the extracellular domain generally results in signal transduction which transmits a biological signal to intracellular targets. Often, this signal transduction acts via a catalytic 40 intracellular domain. The particular array of sequence motifs of this catalytic intracellular domain determines its access to potential kinase substrates (Mohammadi, et al., 1990, Mol. Cell. Biol. 11:5068-5078; Fantl, et al., 1992, Cell 69:413-413). Examples of receptors that transduce signals via cata- 45 lytic intracellular domains include the receptor tyrosine kinases (RTKs) such as the Trk family of receptors which are generally limited to cells of the nervous system, the cytokine family of receptors including the tripartate CNTF receptor complex (Stahl & Yancopoulos, 1994, J. Neurobio. 25:1454-50 1466) which is also generally limited to the cells of the nervous system, G-protein coupled receptors such as the  $\beta_2$ -adrenergic receptor found on, for instance, cardiac muscle cells, and the multimeric IgE high affinity receptor  $Fc \in RI$  which is localized, for the most part, on mast cells and basophils (Sut- 55 ton & Gould, 1993, Nature 366:421-428).

All receptors identified so far appear to undergo dimerization, multimerization, or some related conformational change following ligand binding (Schlessinger, J., 1988, Trend Biochem. Sci. 13:443-447; Ullrich & Schlessinger, 1990, Cell 60 61:203-212; Schlessinger & Ullrich, 1992, Neuron 9:383-391) and molecular interactions between dimerizing intracellular domains lead to activation of catalytic function. In some instances, such as platelet-derived growth factor (PDGF), the ligand is a dimer that binds two receptor mol-65 ecules (Hart, et al., 1988, Science, 240:1529-1531; Heldin, 1989, J. Biol. Chem. 264:8905-8912) while, for example, in

the case of epidermal growth factor (EGF), the ligand is a monomer (Weber, et al., 1984, J. Biol. Chem. 259:14631-14636). In the case of the Fc $\epsilon$ RI receptor, the ligand, IgE, exists bound to Fc $\epsilon$ RI in a monomeric fashion and only becomes activated when antigen binds to the IgE/Fc $\epsilon$ RI complex and cross-links adjacent IgE molecules (Sutton & Gould, 1993, Nature 366:421-428).

Often, the tissue distribution of a particular receptor within higher organisms provides insight into the biological function of the receptor. The RTKs for some growth and differentiation factors, such as fibroblast growth factor (FGF), are widely expressed and therefore appear to play some general role in tissue growth and maintenance. Members of the Trk RTK family (Glass & Yancopoulos, 1993, Trends in Cell Biol. 3:262-268) of receptors are more generally limited to cells of the nervous system, and the Nerve Growth Factor family consisting of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), which bind the Trk RTK family receptors, promote the differentiation of diverse groups of neurons in the brain and periphery (Lindsay, R. M, 1993, in Neurotrophic Factors, S. E. Loughlin & J. H. Fallon, eds., pp. 257-284, San Diego, Calif., Academic Press). FceRI is localized to a very limited number of types of cells such as mast cells and basophils. Mast cells derive from bone marrow pluripotent hematopoietic stem cell lineage, but complete their maturation in the tissue following migration from the blood stream (See Janeway & Travers, 1996, in Immunobiology, 2d. Edition, M. Robertson & E. Lawrence, eds., pp. 1:3-1:4, Current Biology Ltd., London, UK, Publisher) and are involved in the allergic response.

Many studies have demonstrated that the extracellular domain of a receptor provides the specific ligand binding characteristic. Furthermore, the cellular environment in which a receptor is expressed may influence the biological response exhibited upon binding of a ligand to the receptor. For example, when a neuronal cell expressing a Trk receptor is exposed to a neurotrophin which binds to that receptor, neuronal survival and differentiation results. When the same receptor is expressed by a fibroblast, exposure to the neurotrophin results in proliferation of the fibroblast (Glass, et al., 1991, Cell 66:405-413).

A class of cell-derived dimeric mitogens with selectivity for vascular endothelial cells has been identified and designated vascular endothelial cell growth factor (VEGF). VEGF has been purified from conditioned growth media of rat glioma cells (Conn et al., 1990, Proc. Natl. Acad. Sci. U.S.A., 87. pp 2628-2632); and conditioned growth media of bovine pituitary follicle stellate cells (Ferrara and Henzel, 1989, Biochem. Biophys. Res. Comm., 161, pp. 851-858; Gozpadorowicz et al., 1989, Proc. Natl. Acad. Sci. U.S.A., 86, pp. 7311-7315 and conditioned growth medium from human U937 cells (Connolly, D. T. et al. 1989, Science, 246, pp. 1309-1312). VEGF is a dimer with an apparent molecular mass of about 46 kDa with each subunit having an apparent molecular mass of about 23 kDa. VEGF has some structural similarities to platelet derived growth factor (PDGF), which is a mitogen for connective tissue cells but not mitogenic for vascular endothelial cells from large vessels.

The membrane-bound tyrosine kinase receptor, known as Flt, was shown to be a VEGF receptor (DeVries, C. et al., 1992, Science, 255, pp. 989-991). The Flt receptor specifically binds VEGF which induces mitogenesis. Another form of the VEGF receptor, designated KDR, is also known to bind VEGF and induce mitogenesis. The partial cDNA sequence and nearly full length protein sequence of KDR is known as

well (Terman, B. I. et al., 1991 Oncogene 6, pp. 1677-1683; Terman, B. I. et al., 1992 Biochem. Biophys. Res. Comm. 187, pp. 1579-1586).

Persistent angiogenesis may cause or exacerbate certain diseases such as psoriasis, rheumatoid arthritis, hemangiomas, angiofibromas, diabetic retinopathy and neovascular glaucoma. An inhibitor of VEGF activity would be useful as a treatment for such diseases and other VEGF-induced pathological angiogenesis and vascular permeability conditions, such as tumor vascularization. The present invention relates to a VEGF inhibitor that is based on the VEGF receptor Flt1.

Plasma leakage, a key component of inflammation, occurs in a distinct subset of microvessels. In particular, in most organs plasma leakage occurs specifically in the venules. Unlike arterioles and capillaries, venules become leaky in response to numerous inflammatory mediators including histamine, bradykinin, and serotonin. One characteristic of inflammation is the plasma leakage that results from intercellular gaps that form in the endothelium of venules. Most 20 experimental models of inflammation indicate that these intercellular gaps occur between the endothelial cells of postcapillary and collecting venules (Baluk, P., et al., Am. J. Pathol., 1998, 152:1463-76). It has been shown that certain lectins may be used to reveal features of focal sites of plasma 25 leakage, endothelial gaps, and finger-like processes at endothelial cell borders in inflamed venules (Thurston, G., et al., Am. J. Physiol., 1996, 271: H2547-62). In particular, plant lectins have been used to visualize morphological changes at endothelial cell borders in inflamed venules of, for example, the rat trachea. Lectins, such as conconavalin A and ricin, that bind focally to inflamed venules reveal regions of the subendothelial vessel wall exposed by gaps that correspond to sites of plasma leakage (Thurston, G., et al., Am J. Physiol., 1996, 35 (MAbs) are basic, positively charged proteins, and mamma-271: H2547-62).

The properties of the microvessels are dynamic. Chronic inflammatory diseases, for example, are associated with microvascular remodeling, including angiogenesis and microvessel enlargement. Microvessels can also remodel by 40 acquiring abnormal phenotypic properties. In a murine model of chronic airway inflammation, airway capillaries acquire properties of venules, including widened vessel diameter, increased immunoreactivity for von Willebrand factor, and increased immunoreactivity for P-selectin. In addition, these 45 remodeled vessels leak in response to inflammatory mediators, whereas vessels in the same position in the airways of normal mice do not.

Certain substances have been shown to decrease or inhibit vascular permeability and/or plasma leakage. For example, 50 mystixins are synthetic polypeptides that have been reported to inhibit plasma leakage without blocking endothelial gap formation (Baluk, P., et al., J. Pharmacol. Exp. Ther., 1998, 284: 693-9). Also, the beta 2-adrenergic receptor agonist formoterol reduces microvascular leakage by inhibiting 55 endothelial gap formation (Baluk, P. and McDonald, D. M., Am. J. Physiol., 1994, 266:L461-8).

The angiopoietins and members of the vascular endothelial growth factor (VEGF) family are the only growth factors thought to be largely specific for vascular endothelial cells. 60 Targeted gene inactivation studies in mice have shown that VEGF is necessary for the early stages of vascular development and that Ang-1 is required for later stages of vascular remodeling

U.S. Pat. No. 6,011,003, issued Jan. 4, 2000, in the name of 65 Metris Therapeutics Limited, discloses an altered, soluble form of FLT polypeptide being capable of binding to VEGF

and thereby exerting an inhibitory effect thereon, the polypeptide comprising five or fewer complete immunoglobulin domains.

U.S. Pat. No. 5,712,380, issued Jan. 27, 1998 and assigned to Merck & Co., discloses vascular endothelial cell growth factor (VEGF) inhibitors that are naturally occurring or recombinantly engineered soluble forms with or without a C-terminal transmembrane region of the receptor for VEGF.

Also assigned to Merck & Co. is PCT Publication No. WO 98/13071, published Apr. 2, 1998, which discloses gene therapy methodology for inhibition of primary tumor growth and metastasis by gene transfer of a nucleotide sequence encoding a soluble receptor protein which binds to VEGF.

PCT Publication No. WO 97/44453, published Nov. 27, 1997, in the name of Genentech, Inc., discloses novel chimeric VEGF receptor proteins comprising amino acid sequences derived from the vascular endothelial growth factor (VEGF) receptors Flt1 and KDR, including the murine homologue to the human KDR receptor FLK1, wherein said chimeric VEGF receptor proteins bind to VEGF and antagonize the endothelial cell proliferative and angiogenic activity thereof.

PCT Publication No. WO 97/13787, published Apr. 17, 1997, in the name of To a Gosei Co., LTD., discloses a low molecular weight VEGF inhibitor usable in the treatment of diseases accompanied by neovascularization such as solid tumors. A polypeptide containing the first immunoglobulinlike domain and the second immunoglobulin-like domain in the extracellular region of a VEGF receptor FLT but not containing the sixth immunoglobulin-like domain and the seventh immunoglobulin-like domain thereof shows a VEGF inhibitory activity.

Sharifi, J. et al., 1998, The Quarterly Jour. of Nucl. Med. 42:242-249, disclose that because monoclonal antibodies lian cells are negatively charged, the electrostatic interactions between the two can create higher levels of background binding resulting in low tumor to normal organ ratios. To overcome this effect, the investigators attempted to improve MAb clearance by using various methods such as secondary agents as well as chemical and charge modifications of the MAb itself.

Jensen-Pippo, et al., 1996, Pharmaceutical Research 13:102-107, disclose that pegylation of a therapeutic protein, recombinant human granulocyte colony stimulating factor (PEG-G-CSF), results in an increase in stability and in retention of in vivo bioactivity when administered by the intraduodenal route.

Tsutsumi, et al., 1997, Thromb Haemost. 77:168-73, disclose experiments wherein the in vivo thrombopoietic activity of polyethylene glycol-modified interleukin-6 (MPEG-IL-6), in which 54% of the 14 lysine amino groups of IL-6 were coupled with PEG, was compared to that of native IL-6.

Yang, et al., 1995, Cancer 76:687-94, disclose that conjugation of polyethylene glycol to recombinant human interleukin-2 (IL-2) results in a compound, polyethylene glycolmodified IL-2 (PEG-IL-2) that retains the in vitro and in vivo activity of IL-2, but exhibits a markedly prolonged circulating half-life.

R. Duncan and F. Spreafico, Clin. Pharmacokinet., 27: 290-306, 296 (1994) review efforts to improve the plasma half-life of asparaginase by conjugating polyethylene glycol.

PCT International Publication No. WO 99/03996 published Jan. 28, 1999 in the name of Regeneron Pharmaceuticals, Inc. and The Regents of The University of California describes modified human noggin polypeptides having deletions of regions of basic amino acids. The modified human

noggin polypeptides are described as retaining biological activity while having reduced affinity for heparin and superior pharmacokinetics in animal sera as compared to the unmodified human noggin.

## SUMMARY OF THE INVENTION

The present invention is directed to VEGF antagonists with improved pharmacokinetic properties. A preferred embodiment is an isolated nucleic acid molecule encoding a fusion 10 polypeptide capable of binding a VEGF polypeptide comprising (a) a nucleotide sequence encoding a VEGF receptor component operatively linked to (b) a nucleotide sequence encoding a multimerizing component, wherein the VEGF receptor component is the only VEGF receptor component of 15 the fusion polypeptide and wherein the nucleotide sequence of (a) consists essentially of a nucleotide sequence encoding the amino acid sequence of Ig domain 2 of the extracellular domain of a first VEGF receptor and a nucleotide sequence encoding the amino acid sequence of Ig domain 3 of the 20 extracellular domain of a second VEGF receptor.

In a further embodiment, the isolated nucleic acid of the first VEGF receptor is Flt1.

In a further embodiment, the isolated nucleic acid of the second VEGF receptor is Flk1.

In yet another embodiment, the isolated nucleic acid of the second VEGF receptor is Flt4.

In another preferred embodiment, the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the nucleotide sequence 30 encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

In still another preferred embodiment, the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the nucleotide 35 sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

In a preferred embodiment of the invention, the multimerizing component comprises an immunoglobulin domain.

In another embodiment, the immunoglobulin domain is 40 selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include an isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding 45 region of the nucleic acid molecule consists of a nucleotide sequence selected from the group consisting of (a) the nucleotide sequence set forth in FIGS. 13A-13D (SEQ ID No:3); (b) the nucleotide sequence set forth in FIGS. 14A-14C (SEQ ID NO:5); (c) the nucleotide sequence set forth in FIGS. 50 15A-15C (SEQ ID NO:7); (d) the nucleotide sequence set forth in FIGS. 16A-16D (SEQ ID NO:9); (e) the nucleotide sequence set forth in FIGS. 21A-21C (SEQ ID NO:11); (f) the nucleotide sequence set forth in FIGS. 22A-22C (SEQ ID NO:13); (g) the nucleotide sequence set forth in FIGS. 24A- 55 24C; and (SEQ ID NO:15); and (h) a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b), (c), (d), (e), (f), or (g) and which encodes a fusion polypeptide molecule having the biological activity of the modified Flt1 receptor 60 fusion polypeptide.

In a further embodiment of the invention, a fusion polypeptide is encoded by the isolated nucleic acid molecules described above.

A preferred embodiment is a composition capable of bind-65 ing a VEGF molecule to form a nonfunctional complex comprising a multimer of the fusion polypeptide.

Also preferred is a composition wherein the multimer is a dimer.

In yet another embodiment, the composition is in a carrier. Another embodiment is a vector which comprises the

Another embodiment is a vector which comprises the nucleic acid molecules described above, including an expression vector comprising a the nucleic acid molecules described wherein the nucleic acid molecule is operatively linked to an expression control sequence.

Other included embodiments are a host-vector system for the production of a fusion polypeptide which comprises the expression vector, in a suitable host cell; the host-vector system wherein the suitable host cell is a bacterial cell, yeast cell, insect cell, or mammalian cell; the host-vector system wherein the suitable host cell is *E. coli*; the host-vector system wherein the suitable host cell is a COS cell; the host-vector system wherein the suitable host cell is a CHO cell.

Another embodiment of the invention is a method of producing a fusion polypeptide which comprises growing cells of the host-vector system under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

Additional embodiments include a fusion polypeptide encoded by the nucleic acid sequence set forth in FIGS. 25 **10A-10D** (SEQ ID NO:1) or FIGS. **24A-24**C (SEQ ID NO:15), which has been modified by acetylation or pegylation wherein the acetylation is accomplished with at least about a 100 fold molar excess of acetylation reagent or wherein acetylation is accomplished with a molar excess of 30 acetylation reagent ranging from at least about a 10 fold molar excess to about a 100 fold molar excess or wherein the pegylation is 10K or 20K PEG.

A preferred embodiment includes a method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal the fusion polypeptide described above, including embodiments wherein the mammal is a human, the fusion polypeptide is acetylated or the fusion polypeptide is pegylated.

A further embodiments is a fusion polypeptide which specifically binds the VEGF receptor ligand VEGF.

A preferred embodiment of the invention is a method of blocking blood vessel growth in a human comprising administering an effective amount of the fusion polypeptide described above.

Also preferred is a method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of the fusion polypeptide described above.

Preferred embodiments of these methods are wherein the mammal is a human.

Further embodiments of the methods of the invention include attenuation or prevention of tumor growth in a human; attenuation or prevention of edema in a human, especially wherein the edema is brain edema; attenuation or prevention of ascites formation in a human, especially wherein the ascites is ovarian cancer-associated ascites.

Preferred embodiments of the invention include a fusion polypeptide capable of binding a VEGF polypeptide comprising (a) a VEGF receptor component operatively linked to (b) a multimerizing component, wherein the VEGF receptor component is the only VEGF receptor component in the fusion polypeptide and consists essentially of the amino acid sequence of Ig domain 2 of the extracellular domain of a first VEGF receptor and the amino acid sequence of Ig domain 3 of the extracellular domain of a second VEGF receptor.

In a further embodiment of the fusion polypeptide, the first VEGF receptor is Flt1.

In yet a further embodiment of the fusion polypeptide, the second VEGF receptor is Flk1.

Still another embodiment of the fusion polypeptide is one in which the second VEGF receptor is Flt4.

Preferred embodiments include a fusion polypeptide 5 wherein amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor and a fusion polypeptide wherein the amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor.

In yet another embodiment, the fusion polypeptide multimerizing component comprises an immunoglobulin domain including an embodiment wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include a fusion polypeptide com- 20 prising an amino acid sequence of a modified Flt1 receptor, wherein the amino acid sequence selected from the group consisting of (a) the amino acid sequence set forth in FIGS. 13A-13D (SEQ ID NO:4); (b) the amino acid sequence set forth in FIGS. 14A-14C (SEQ ID NO:6); (c) the amino acid 25 sequence set forth in FIGS. 15A-15C (SEQ ID NO:8); (d) the amino acid sequence set forth in FIGS. 16A-16D (SEQ ID NO:10); (e) the amino acid sequence set forth in FIGS. 21A-21C (SEQ ID NO:12); (f) the amino acid sequence set forth in FIGS. 22A-22C (SEQ ID NO:14); and (g) the amino acid -30 sequence set forth in FIGS. 24A-24C (SEQ ID NO:16).

Another preferred embodiment is a method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal the fusion polypeptide described above.

An alternative preferred embodiment is a method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of the fusion polypeptide described above.

#### BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. IEF gel analysis of unmodified and acetylated Flt1(1-3)-Fc proteins. Unmodified Flt1(1-3)-Fc protein is unable to enter the gel due to its >9.3 pI, whereas acetylated 45 Flt1(1-3)-Fc is able to enter the gel and equilibrate at pI 5.2.

FIG. 2. Binding of unmodified Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc proteins to MATRIGEL® coated plates. Unmodified Flt1(1-3)-Fc proteins binds extensive to extracellular matrix components in MATRIGEL®, whereas acety- 50 lated Flt1(1-3)-Fc does not bind.

FIG. 3. Binding of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc in a BIACORE[™]based assay. Acetylated (columns 13-16), pegylated (columns 17-20), and heparin-treated Flt1(1-3)-Fc (columns 55 21-24) are each able to completely compete with the BIA-CORE™ chip-bound Flt1(1-3)-Fc for VEGF binding as compared to control (columns 1-4) and irrelevant protein (columns 5-8). Unmodified Flt1(1-3)-Fc (columns 5-6) appears to only partially compete with BIACORE™ chip-bound Flt1 60 (1-3)-Fc for VEGF binding. However, washing the bound samples with 0.5 M NaCl (columns 7-8) results in a binding profile similar to the modified forms of Flt1(1-3)-Fc, indicating that the unmodified protein is exhibiting non-specific binding to the chip that can be eliminated by the salt wash. 65

FIG. 4. Binding of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc to VEGF in an

ELISA-based assay. Both pegylated and acetylated Flt1(1-3)-Fc proteins bind to VEGF with affinities approaching that of unmodified Flt1(1-3)-Fc.

FIG. 5. Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc. Balb/c mice (23-28 g) were injected subcutaneously with 4 mg/kg of unmodified, acetylated, or pegylated Flt1(1-3)-Fc. The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days, and 3 days after injection of protein and the sera were assayed in a standard ELISA-based assay designed to detect Flt1(1-3)-Fc protein. The  $T_{max}$  for all of the Flt1(1-3)-Fc proteins was between the 6 hour and 24 hour time points. The  $C_{max}$  for the different proteins was as follows: Unmodified: 0.06 µg/ml-0.15  $\mu g/ml;$  acetylated: 1.5  $\mu g/ml\text{-}4.0$   $\mu g/ml;$  and pegylated: approximately 5 µg/ml.

FIGS. 6A-6B. IEF gel analysis of unmodified and stepacetylated Flt1(1-3)-Fc proteins. Unmodified Flt1(1-3)-Fc protein is unable to enter the gel due to its >9.3 pI, whereas most of the step-acetylated Flt1(1-3)-Fc samples (30-100 fold excess samples) were able to migrate into the gel and equilibrate at pIs ranging between 4.55-8.43, depending on the degree of acetylation.

FIG. 7. Binding of unmodified Flt1(1-3)-Fc and stepacetylated Flt1(1-3)-Fc proteins to MATRIGEL® coated plates. As with the irrelevant control protein, rTie2-Fc, stepacetylated Flt1(1-3)-Fc (20 and 30 fold excess samples) does not exhibit any binding to the MATRIGEL® coated plate, whereas the non-acetylated Flt1(1-3)-Fc protein exhibits significant binding. The 10 fold excess sample shows reduced binding, but the degree of acetylation is not enough to completely block binding to extracellular matrix components.

FIG. 8. Binding of unmodified Flt1(1-3)-Fc and stepacetylated Flt1(1-3)-Fc in a BIACORE[™]-based assay. At a 35 sub-stoichiometric ratio (0.5 µg/ml of either unmodified Flt1 (1-3) or step-acetylated Flt1(1-3)-Fc vs. 0.2 µg/ml VEGF), there is not enough Flt1(1-3)-Fc (either unmodified or stepacetylated) in the solution to completely bind the VEGF. At 1.0 µg/ml, which approximates a 1:1 stoichiometric ratio, the 40 both unmodified and step-acetylated Flt1(1-3)-Fc are better able to compete for VEGF binding, but there is still insufficient Flt1(1-3)-Fc protein (either unmodified or step-acetylated) to completely saturate the available VEGF. However, at 5.0 µg/ml, which is several times greater than a 1:1 stoichiometric ratio, both the Flt1(1-3)-Fc and the step-acetylated Flt1(1-3)-Fc proteins are able to saturate the VEGF, regardless of the degree of acetylation.

FIG. 9. Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc. Balb/c mice (23-28 g) were injected subcutaneously with 4 mg/kg of unmodified or 10, 20, 40, 60 and 100 fold excess samples of step-acetylated Flt1(1-3)-Fc (3 mice for unmodified, 10, 20 and 40 fold excess samples and 2 mice for 60 and 100 fold excess samples). The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days and 3 days after injection. The sera were assayed in an ELISA-based assay designed to detect Flt1(1-3)-Fc. The  $T_{max}$ for all of the Flt1(1-3)-Fc proteins tested was at the 6 hour time point but the Cmax was as follows: Unmodified Flt1(1-3)-Fc: 0.06 µg/ml; 10 fold excess sample: -0.7 µg/ml, 20 fold excess sample-2 µg/ml, 40 fold excess sample-4 µg/ml, 60 fold excess sample-2 µg/ml, 100 fold excess sample-1 µg/ml.

FIGS. 10A-10D. Nucleic acid (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of Flt1(1-3)-Fc.

FIG. 11. Schematic diagram of the structure of Flt1.

FIGS. 12A and 12B. Hydrophilicity analysis of the amino acid sequences of Ig domain 2 and Ig domain 3 of Flt1.

FIGS. **13**A-**13**D. Nucleic acid (SEQ ID NO:3) and deduced amino acid sequence (SEQ ID NO:4) of Mut1: Flt1  $(1-3_{AB})$ -Fc.

FIGS. **14**A-**14**C. Nucleic acid (SEQ ID NO:5) and deduced amino acid sequence (SEQ ID NO:6) of Mut2: Flt1(2- $3_{\Delta B}$ )-Fc.

FIGS. **15**A-**15**C. Nucleic acid (SEQ ID NO:7) and deduced amino acid sequence (SEQ ID NO:8) of Mut3: Flt1(2-3)-Fc.

FIGS. **16A-16**D. Nucleic acid (SEQ ID NO:9) and deduced amino acid sequence (SEQ ID NO: 10) of Mut4: 10  $Flt1(1-3_{R->N})$ -Fc.

FIG. 17. Binding of unmodified Flt1(1-3)-Fc, basic region deletion mutant Flt1(1-3)-Fc, and Flt1(1-3)_{*R*->*N*} mutant proteins in a BIACORETM-based assay. At the sub-stoichiometric ratio (0.25 µg/ml Flt1(1-3)-Fc of unmodified, acetylated or genetically modified samples vs. 01. µg/ml VEGF), there is insufficient Flt1(1-3)-Fc protein to block binding of VEGF to the Flt1(1-3)-Fc immobilized on the BIACORE™ chip. At 0.5 µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, the stoichiometric ratio approximates 20 1:1 and there is an increased ability to block VEGF binding to the BIACORETM chip. At 1.0 µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, which is approximately a 10:1 stoichiometric ratio, the Flt1(1-3)-Fc proteins are able to block binding of VEGF to the BIA- 25 CORE™ chip, but they are not equivalent. Unmodified, acetylated, and Mut1: Flt1(1-3_{$\Delta B$})-Fc are essentially equal in their ability to block VEGF binding, whereas Mut4: Flt1  $(1-3_{R->N})$ -Fc is somewhat less efficient at blocking binding.

FIG. 18. Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1 30  $(1-3_{\Delta B})$ -Fc, Mut2: Flt1(2-3_{\Delta B})-Fc, and Flt1(2-3) mutant proteins to MATRIGEL® coated plates. Unmodified Flt1(1-3)-Fc protein binds avidly to these wells, the Mut3: Flt1(2-3)-Fc protein binds somewhat more weakly, the Mut1: Flt1 (1-3_{\Delta B})-Fc protein binds more weakly still, and the Mut2: Flt1(2- 35  $3_{\Delta B}$ )-Fc protein shows the best profile, binding more weakly than any of the other mutant proteins. The Mut4: Flt1  $(1-3_{R-N})$ -Fc glycosylation mutant protein shows only marginal benefit on the MATRIGEL® assay.

FIG. **19**. Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1 40  $(1-3_{\Delta B})$ -Fc, Mut2: Flt1(2-3_{\Delta B})-Fc, and Flt1(2-3) mutant proteins in an ELISA-based assay. At the concentrations tested, unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3_{\Delta B})-Fc, Mut2: Flt1  $(2-3_{\Delta B})$ -Fc, and Flt1(2-3) mutant proteins bind VEGF similarly. 45

FIG. **20**. Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3_{$\Delta B$})-Fc, Mut2: Flt1(2-3_{$\Delta B$})-Fc, and Flt1 (2-3) mutant proteins. the C_{max} for these reagents was as follows: Unmodified Flt1(1-3)-Fc-0.15 µg/ml; 40 fold molar excess acetylated Flt1(1-3)-Fc-1.5 µg/ml; and Mut1: Flt1(1- 50 3_{$\Delta B$})-Fc-0.7 µg/ml.

FIGS. **21**Å-**21**C. Nucleotide (SEQ ID NO:11) and deduced amino acid sequence (SEQ ID NO:12) of the modified Flt1 receptor termed Flt1D2.Flk1D3.Fc $\Delta$ G1(a).

FIGS. **22**A-**22**C. Nucleotide (SEQ ID NO:13) and deduced 55 amino acid sequence (SEQ ID NO:14) of the modified Flt1 receptor termed Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a).

FIG. 23. Extracellular Matrix (ECM) Assay. The results of this assay demonstrate that the Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a) proteins are considerably less 60 sticky to the ECM as compared to the Flt1(1-3)-Fc protein.

FIGS. **24**A-**24**C. Nucleotide (SEQ ID NO: 15) and deduced amino acid sequence (SEQ ID NO: 16) of the modified Flt1 receptor termed VEGFR1R2-Fc $\Delta$ C1(a).

FIGS. **25**A-**25**C. Phosphorylation assay. At a 1.5 molar 65 excess of either Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3.Fc $\Delta$ C1(a) there is complete blockage of

receptor stimulation by these three modified Flt1 receptors as compared to control media challenge. In contrast, transient Flt1D2VEGFR3D3.Fc $\Delta$ C1(a) does not show significant blockage at this molar excess, as compared with VEGF positive control challenge. Similar results are seen in FIG. **25**B, where the modified Flt receptors are in a 3-fold molar excess to VEGF165 ligand. In FIG. **25**C, where the modified Flt1 receptors are in a 6-fold molar excess to VEGF165 ligand, transient Flt1D2VEGFR3D3.Fc $\Delta$ C1(a) can now be shown to be partially blocking VEGF165-induced stimulation of cell-surface receptors.

FIGS. **26**A-**26**B. Phosphorylation assay. Detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are not phosphorylated by challenge samples which have VEGF165 preincubated with 1 and 2 fold molar excess (FIG. **26**A) or 3 and 4 fold molar excess (FIG. **26**B) of either transient Flt1D2Flk1D3.Fc $\Delta$ C1(a), stable Flt1D2Flk1D3.Fc $\Delta$ C1(a), or transient VEGFR1R2—Fc $\Delta$ C1 (a). At all modified Flt1 receptor concentrations tested there is complete binding of VEGF165 ligand during the preincubation, resulting in no detectable stimulation of cell-surface receptors by unbound VEGF165 as compared to control media challenge.

FIG. 27. MG/R2 Cell proliferation assay. The following modified Flt receptors Flt1(1-3)-Fc, Flt1D2.Flk1D3.Fc $\Delta$ C1 (a) and Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a), plus an irrelevant receptor termed Tie2-Fc as a negative control, were titrated from 40 nM to 20  $\mu$ M and incubated on the cells for 1 hr at 37° C. Human recombinant VEGF165 in defined media was then added to all the wells at a concentration of 1.56 nM. The negative control receptor Tie2-Fc does not block VEGF165-induced cell proliferation at any concentration whereas Flt1D2.Flk1D3.Fc $\Delta$ C1(a) blocks 1.56 nM VEGF165 with a half maximal dose of 0.8 nM. Flt1(1-3)-Fc and Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a) are less effective in blocking VEGF165 in this assay with a half maximal dose of -2 nM. VEGF165 alone gives a reading of 1.2 absorbance units and the background is 0.38 absorbance units.

FIG. **28**. BIACORETM analysis of Binding Stoichiometry. Binding stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1 D2Flk1D3.Fc $\Delta$ C1 (a) or VEGFR1R2-Fc $\Delta$ C1(a), using the conversion factor of 1000 RU equivalent to 1 ng/ml. The results indicated binding stoichiometry of one VEGF165 dimeric molecule per one Flt1D2Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a) molecule.

FIG. 29 and FIG. 30. Size Exclusion Chromatography Stoichiometry. Flt1D2Flk1D3.Fc AC1(a) or VEGFR1R2- $Fc\Delta C1(a)$  at a concentration of 1 nM (estimated to be 1000 times higher than the KD of the Flt1D2Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a)/VEGF165 interaction) were mixed with varied concentrations of VEGF165. After incubation, concentrations of the free Flt1D2Flk1D3.Fc $\Delta$ C1(a) in solution were measured. The data shows that the addition of 1 nM VEGF165 into the Flt1D2Flk1D3.Fc∆C1(a) solution completely blocks Flt1D2Flk1D3.Fc\DeltaC1(a) binding to the VEGF165 surface. This result suggested the binding stoichiometry of one VEGF165 molecule per one Flt1D2Flk1D3.FcΔC1(a) molecule.

FIG. **31**. Size Exclusion Chromatography (SEC) under native conditions. Peak #1 represents the Flt1D2Flk1D3.Fc $\Delta$ C1(a)/VEGF165 complex and peak #2 represents unbound VEGF165. Fractions eluted between 1.1 and 1.2 ml were combined and guanidinium hydrochloride (GuHCl) was added to a final concentration 4.5 M to dissociate the complex.

FIG. 32. Size Exclusion Chromatography (SEC) under dissociative conditions. To separate the components of the receptor-ligand complex and to determine their molar ratio, 50 µl of dissociated complex was loaded onto a SUPER-OSE™ 12 PC 3.2/30 equilibrated in 6 M GuHCl and eluted. 5 Peak #1 represents Flt1D2Flk1D3.Fc\DeltaC1(a) and peak #2 represents VEGF165.

FIG. 33, FIG. 34 and FIG. 35. Size Exclusion Chromatography (SEC) with On-Line Light Scattering. Size exclusion chromatography column with a MiniDawn on-line light scat- 10 tering detector (Wyatt Technology, Santa Barbara, Calif.) and refractive index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine the molecular weight (MW) of the receptor-ligand complex. As shown in FIG. 33, the elution profile shows two peaks. Peak #1 represents the receptor-ligand 15 complex and peak #2 represents the unbound VEGF165. MW was calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows: MW of the Flt1 D2Flk1D3.Fc\DeltaC1(a)/ 20 VEGF165 complex at the peak position is 157 300 (FIG. 33), the MW of VEGF165 at the peak position is 44 390 (FIG. 34) and the MW of R1R2 at the peak is 113 300 (FIG. 35).

FIG. 36. Peptide mapping and glycosylation analysis. The disulfide structures and glycosylation sites in 25 Flt1D2.Flk1D3.Fc\DeltaC1(a) (SEQ ID NO:12) were determined by a peptide mapping method. There are a total of ten cysteines in Flt1D2.Flk1D3.Fc $\Delta$ C1(a); six of them belong to the Fc region. Cys27 is disulfide bonded to Cys76. Cys121 is disulfide bonded to Cys182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two cysteines in another Fc chain. However, it can not be determined whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is disul- 35 fide bonded to Cys306. Cys352 is disulfide bonded to Cys410.

There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.Fc\DeltaC1(a) (SEQ ID NO:12) and are found to be glycosylated to varying degrees. Complete glycosylation 40 is observed at Asn33, Asn193, and Asn282. Partial glycosylation is observed on Asn65 and Asn120. Sites of glycosylation are highlighted by underline in the Figure.

FIG. 37. Pharmacokinetics of Flt1(1-3)-Fc (A40),  $Flt1D2.Flk1D3.Fc\DeltaC1(a)$  and  $VEGFR1R2-Fc\DeltaC1(a)$ . 45 receptor based VEGF antagonist that has a pharmacokinetic Balb/c mice were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.Fc\DeltaC1(a), CHO stably expressed Flt1D2. Flk1D3.Fc $\Delta$ C1(a), and CHO transiently expressed VEGFR1R2-Fc $\Delta$ C1(a). The mice were tail bled at 1, 2, 4, 6, 50 24 hrs, 2 days, 3 days and 6 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a). The  $T_{max}$  for Flt1(1-3)-Fc (A40) was at 6 hrs while the  $T_{max}$  for the transient and stable Flt1D2.Flk1D3.Fc∆C1(a) and the tran- 55 sient VEGFR1R2-Fc $\Delta$ C1(a) was 24 hrs. The C_{max} for Flt1(1-3)-Fc (A40) was 8 µg/ml, For both transients (Flt1D2. Flk1D3.Fc $\Delta$ C1(a) and VEGFR1R2-Fc $\Delta$ C1(a)) the C_{max} was 18 µg/ml and the  $C_{max}$  for the stable VEGFR1R2-Fc $\Delta$ C1(a) was 30 µg/ml.

FIG. 38. Pharmacokinetics of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1 (a). Balb/c mice were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.Fc\DeltaC1(a) and CHO transiently expressed 65 Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a). The mice were tail bled at 1, 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were

assayed in an ELISA designed to detect Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1 (a). Flt1(1-3)-Fc (A40) could no longer be detected in the serum after day 5 whereas Flt1D2.Flk1D3.Fc∆C1(a) and Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a) were detectable for 15 days or more

FIG. 39. The Ability of Flt1D2.Flk1D3.Fc $\Delta$ C1(a) to Inhibit HT-1080 Fibrosarcoma Tumor Growth In Vivo. Every other day or 2 times per week treatment of SCID mice with Flt1D2.Flk1D3.FcΔC1(a) at 25 mg/Kg significantly decreases the growth of subcutaneous HT-1080 fibrosarcoma tumors

FIG. 40. The Ability of Flt1D2.Flk1D3.FcΔC1(a) to Inhibit C6 Glioma Tumor Growth In Vivo. Every other day or 2 times a week treatment of SCID mice with Flt1 D2.Flk1D3.Fc $\Delta$ C1(a) significantly decreases the growth of subcutaneous C6 glioma tumors at doses as low as 2.5 mg/Kg.

FIG. 41. VEGF-Induced Uterine Hyperpermeability. Pregnant mare's serum gonadotrophin (PMSG) injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats results in a surge of estradiol after 2 days which in turn causes an induction of VEGF in the uterus. This induction results in hyperpermeability of the uterus and an increase in uterine wet. Subcutaneous injection of Flt1(1-3)-Fc (A40), Flt1 D2.Flk1D3.Fc\DeltaC1(a) and Flt1D2.VEGFR3D3.Fc\DeltaC1(a) at 25 mg/kg at 1 hr after PMSG injection results in about a 50% inhibition of the increase in uterine wet weight.

FIGS. 42A-42B. Assessment of Corpus Luteum Angiogenesis Using Progesterone as a Readout. PMSG was injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats, resulting in a fully functioning corpus luteum containing a dense network of blood vessels that secretes progesterone into the blood stream to prepare the uterus for implantation. The induction of angiogenesis in the corpus luteum requires VEGF. Resting levels of progesterone are about 5 ng/ml and can be induced to 25-40 ng/ml after PMSG. Subcutaneous injection of Flt1(1-3)-Fc (A40) or Flt1D2. Flk1D3.FcΔC1(a) at 25 mg/kg or 5 mg/kg at 1 hr after PMSG injection resulted in a complete inhibition of the progesterone induction on day 4.

# DETAILED DESCRIPTION OF THE INVENTION

It has been a long standing problem in the art to produce a profile that is appropriate for consideration of the antagonist as a therapeutic candidate. Applicants describe herein, for the first time, a chimeric polypeptide molecule, capable of antagonizing VEGF activity, that exhibits improved pharmacokinetic properties as compared to other known receptorbased VEGF antagonists. The chimeric polypeptide molecules described herein thus provide for the first time appropriate molecules for use in therapies in which antagonism of VEGF is a desired result.

The present invention provides for novel chimeric polypeptide molecules formed by fusing a modified extracellular ligand binding domain of the Flt1 receptor to the Fc region of IgG.

The extracellular ligand binding domain is defined as the 60 portion of a receptor that, in its native conformation in the cell membrane, is oriented extracellularly where it can contact with its cognate ligand. The extracellular ligand binding domain does not include the hydrophobic amino acids associated with the receptor's transmembrane domain or any amino acids associated with the receptor's intracellular domain. Generally, the intracellular or cytoplasmic domain of a receptor is usually composed of positively charged or polar

amino acids (i.e. lysine, arginine, histidine, glutamic acid, aspartic acid). The preceding 15-30, predominantly hydrophobic or a polar amino acids (i.e. leucine, valine, isoleucine, and phenylalanine) comprise the transmembrane domain. The extracellular domain comprises the amino acids that 5 precede the hydrophobic transmembrane stretch of amino acids. Usually the transmembrane domain is flanked by positively charged or polar amino acids such as lysine or arginine. von Heijne has published detailed rules that are commonly referred to by skilled artisans when determining which amino acids of a given receptor belong to the extracellular, transmembrane, or intracellular domains (See von Heijne, 1995, BioEssays 17:25-30). Alternatively, websites on the Internet have become available to provide protein chemists with information about making predictions about protein domains.

The present invention provides for the construction of nucleic acid molecules encoding chimeric polypeptide molecules that are inserted into a vector that is able to express the chimeric polypeptide molecules when introduced into an appropriate host cell. Appropriate host cells include, but are 20 not limited to, bacterial cells, yeast cells, insect cells, and mammalian cells. Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding the chimeric polypeptide molecules under control of transcriptional/trans- 25 lational control signals. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination) (See Sambrook, et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory; Current Protocols in Molecular Biology, Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY). Expression of nucleic acid molecules encoding the chimeric polypeptide molecules may be regulated by a second nucleic acid sequence so that the chimeric polypeptide molecule is expressed in a host transformed with the recombinant 35 DNA molecule. For example, expression of the chimeric polypeptide molecules described herein may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control expression of the chimeric polypeptide molecules include, but are not limited to, the long 40 terminal repeat as described in Squinto et al., (1991, Cell 65:1-20); the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the CMV promoter, the M-MuLV 5' terminal repeat the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et 45 al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory sequences of the metallothionine gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the  $\beta$ -lactamase promoter 50 (VIIIa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25, see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94); promoter elements from yeast or other 55 fungi such as the Gal 4 promoter, the ADH (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I 60 gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984,

Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Shani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Thus, according to the invention, expression vectors capable of being replicated in a bacterial or eukaryotic host comprising chimeric polypeptide molecule-encoding nucleic acid as described herein, are used to transfect the host and thereby direct expression of such nucleic acids to produce the chimeric polypeptide molecules, which may then be recovered in a biologically active form. As used herein, a biologically active form includes a form capable of binding to VEGF.

Expression vectors containing the chimeric nucleic acid molecules described herein can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to the inserted chimeric polypeptide molecule sequences. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the chimeric polypeptide molecule DNA sequence is inserted within the marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the chimeric polypeptide molecules.

Cells of the present invention may transiently or, preferably, constitutively and permanently express the chimeric polypeptide molecules.

The chimeric polypeptide molecules may be purified by any technique which allows for the subsequent formation of a stable, biologically active chimeric polypeptide molecule. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8 M guanidinium hydrochloride and dialysis (see, for example, Builder, et al., U.S. Pat. No. 5,663,304). In order to further purify the factors, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

In one embodiment of the invention, the nucleotide sequence encoding the first component is upstream of the

nucleotide sequence encoding the second component. In another embodiment of the invention, the nucleotide sequence encoding the first component is downstream of the nucleotide sequence encoding the second component. Further embodiments of the invention may be prepared in which the order of the first, second and third fusion polypeptide components are rearranged. For example, if the nucleotide sequence encoding the first component is designated 1, the nucleotide sequence encoding the second component is designated 2, and the nucleotide sequence of the third component 10 is designated 3, then the order of the components in the isolated nucleic acid of the invention as read from 5' to 3' may be any of the following six combinations: 1,2,3; 1,3,2; 2,1,3; 2,3,1; 3,1,2; or 3,2,1.

The present invention also has diagnostic and therapeutic utilities. In particular embodiments of the invention, methods of detecting aberrancies in the function or expression of the chimeric polypeptide molecules described herein may be used in the diagnosis of disorders. In other embodiments, manipulation of the chimeric polypeptide molecules or ago-20 nists or antagonists which bind the chimeric polypeptide molecules may be used in the treatment of diseases. In further embodiments, the chimeric polypeptide molecule is utilized as an agent to block the binding of a binding agent to its target.

By way of example, but not limitation, the method of the 25 invention may be useful in treating clinical conditions that are characterized by vascular permeability, edema or inflammation such as brain edema associated with injury, stroke or tumor; edema associated with inflammatory disorders such as psoriasis or arthritis, including rheumatoid arthritis; asthma; 30 generalized edema associated with burns; ascites and pleural effusion associated with tumors, inflammation or trauma; chronic airway inflammation; capillary leak syndrome; sepsis; kidney disease associated with increased leakage of protein; and eye disorders such as age related macular degenera- 35 tion and diabetic retinopathy.

An amino acid sequence analysis of Flt1(1-3)-Fc revealed the presence of an unusually high number (46) of the basic amino acid residue lysine. An IEF analysis of Flt1(1-3)-Fc showed that this protein has pI greater than 9.3, confirming 40 the prediction that the protein is very basic. It was hypothesized that the basic nature of Flt1(1-3)-Fc protein was causing it to bind to extracellular matrix components and that this interaction might be the cause of the extremely short detectable circulating serum half-life exhibited by Flt1(1-3)-Fc 45 when injected into mice. In order to test this hypothesis, Flt1(1-3)-Fc protein was acetylated at the lysine residues to reduce the basic charge. Acetylated Flt1(1-3)-Fc was then tested in the assays described infra.

and not by way of limitation.

## EXAMPLES

# Example 1

#### Expression of Flt1(1-3)-Fc Protein in CHO K1 Cells

Using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., 60 Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-interscience, N.Y.), the gene encoding Flt1(1-3)-Fc was inserted into the expression vector pEE14.1 (Lonza Biologics, plc) at a multiple cloning site downstream of the CMV promoter. CHO K1 cells were transfected with the pEE14.1/ Flt1(1-3)-Fc DNA construct using lipofectamine (Gaithers16

burg, Md.). The transfected CHO K1 cells were grown in glutamine-free DMEM (JRH, Kansas City, Mo.) containing 25 µM methionine sulfoximine (MSX) from Sigma Inc., St. Louis, Mo., and high recombinant protein expressors were obtained by screening the CHO K1 cell supernatants from over 100 hand-picked colony isolates using a standard immunoassay which captures and detects human Fc. The selected hand-picked clone was amplified in the presence of 100  $\mu M$ MSX followed by a second round of screening of the amplified clones. The highest producing clone had a specific productivity of recombinant Flt1(1-3)-Fc protein of 55 µg/cell/ day.

The selected clone was expanded in 225 cm² T-flasks (Corning, Acton, Mass.) and then into 8.5 L roller bottles (Corning, Acton, Mass.) using the cell culture media described supra. Cells were removed from the roller bottles by standard trypsinization and put into 3.5 L of suspension medium. The suspension medium is comprised of glutaminefree ISCHO medium (Irvine Scientific, Santa Ana, Calif.) containing 5% fetal bovine serum (FBS from HYCLONE™ Labs, Logan, Utah), 100 µM MSX and GS supplement (JRH Scientific, Kansas City, Mo.) in a 5 L Celligen bioreactor (New Brunswick Scientific, New Brunswick, N.J.) at a density of  $0.3 \times 10^6$  cells/mL. After the cells reached a density of  $3.6 \times 10^6$ /mL and were adapted to suspension they were transferred to a 60 L bioreactor (ABEC, Allentown, Pa.) at a density of 0.5×10⁶ cells/mL in 20 L of ISCHO medium with 5% fetal bovine serum. After two days an additional 20 L of ISCHO+5% fetal bovine serum was added to the bioreactor. The cells were allowed to grow for an additional two days reaching a final density of  $3.1 \times 10^6$  cells/mL, and a final Flt1 (1-3)-Fc concentration at harvest was 95 mg/L. At harvest the cells were removed by tangential flow filtration using 0.45 µm Prostak Filters (Millipore, Inc., Bedford, Mass.).

## Example 2

# Purification of Flt1(1-3)-Fc Protein Obtained from CHO K1 Cells

Flt1(1-3)-Fc protein was initially purified by affinity chromatography. A Protein A column was used to bind, with high specificity, the Fc portion of the molecule. This affinity-purified protein was then concentrated and passed over a SEC column. The protein was then eluted into the formulation buffer.

The following describes these procedures in detail.

Materials and Methods

All chemicals were obtained from J. T. Baker, Phillipsburg, The following examples are offered by way of illustration 50 N.J. with the exception of PBS, which was obtained as a  $10 \times$ concentrate from Life Technologies, Gaithersburg, Md. Protein A Fast Flow and SUPERDEX™ 200 preparation grade resins were obtained from Pharmacia, Piscataway, N.J. Equipment and membranes for protein concentration were 55 obtained from Millipore, Bedford, Mass.

> Approximately 40 L of 0.45 µm-filtered CHO conditioned media containing Flt1(1-3)-Fc protein was applied to a 290 mL Protein A Fast Flow column (10 cm diameter) that had been equilibrated with PBS. The column was washed with PBS containing 350 mM NaCl and 0.02% CHAPS and the bound protein was eluted with 20 mM Citric Acid containing 10 mM Na₂HPO₄. The single peak in the elution was collected and its pH was raised to neutrality with 1 M NaOH. The eluate fractions was concentrated to approximately 9 mg/mL using 10K regenerated cellulose membranes by both tangential flow filtration and by stirred cell concentration. To remove aggregates and other contaminants, the concentrated protein

was applied to a column packed with SUPERDEXTM 200 preparation grade resin (10 cm×55 cm) and run in PBS containing 5% glycerol. The main peak fractions were pooled, sterile filtered, aliquoted and stored at  $-80^{\circ}$  C.

#### Example 3

#### Acetylation of Flt1(1-3)-Fc Protein

Two milligrams of Flt1(1-3)-Fc protein were acetylated as ¹⁰ described in the instruction manual provided with the sulfo-NHS-acetate modification kit (Pierce Chemical Co., Rockford, Ill., Cat.#26777).

#### Example 4

# Characterization of Acetylated Flt1(1-3)-Fc Protein

IEF analysis: Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc were analyzed by standard IEF analysis. As shown in FIG. 1, ²⁰ Flt1(1-3)-Fc protein is not able to migrate into the gel and therefore must have a pI greater than 9.3, the highest pI in the standard. However, acetylated Flt1(1-3)-Fc is able to migrate into the gel and equilibrate at a pI of approximately 5.2. This result demonstrates that acetylation reduces the net positive ²⁵ charge of the protein and therefore its pi considerably.

Binding to Extracellular Matrix Components

To test for binding to extracellular matrix components, Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc where tested in an assay designed to mimic the interaction with extracellular 30 matrix components. In this assay, 96-well tissue culture plates are coated with MATRIGEL® (Biocoat MATRIGEL® matrix thin layer 96 well plate, Catalog #40607, Becton Dickinson Labware, Bedford, Mass.). The plates are incubated with varying concentrations of either Flt1(1-3)-Fc, acetylated 35 Flt1(1-3)-Fc, or rTie2-Fc (an irrelevant control) protein are added to the wells. The plates are incubated for 1-2 hours at either room temperature or 37° C. degrees and then detection of bound proteins is accomplished by adding a secondary alkaline phosphatase-conjugated anti-human Fc antibody to 40 the wells. Finally, alkaline phosphatase substrate is added to the wells and optical density is measured. FIG. 2 shows the results of this assay. Like the irrelevant control protein rTie2-Fc, acetylated Flt1(1-3)-Fc does not exhibit any binding to the MATRIGEL® coated plate, whereas the non-acetylated Flt1 45 (1-3)-Fc protein exhibits significant binding. This result indicates that acetylation of basic amino acid residues is an effective way to interfere with the charge interactions that exist between positively charged proteins and the negatively charged extracellular matrix components they are exposed to 50 in vivo.

#### Example 5

# Pegylation of Flt1(1-3)-Fc Protein

Although pegylation (polyethylene glycol—PEG) of proteins has been shown to increase their in vivo potency by enhancing stability and bioavailability while minimizing immunogenicity (see references cited supra), it is counter-60 intuitive that pegylating molecules that are too large to be filtered by the kidney glomeruli would improve their pharmacokinetic properties. Without being bound by theory, Applicants postulated that pegylation of the Flt1(1-3)-Fc molecules could improve the pharmacokinetic properties, possibly not 5 by altering the positive charge or by decreasing the pl of Flt1(1-3)-Fc, but rather by physically shielding the positive

charges from interacting with the extracellular matrix. Applicants decided to attempt to improve the pharmacokinetic properties of Flt1(1-3)-Fc molecules by attaching strands of 20K PEGs as described infra.

5 Materials and Methods

Purified Flt1(1-3)-Fc derived from CHO cells (see supra) was used in the following pegylation experiments. Functionalized PEGs were obtained from Shearwater Polymers, Huntsville, Ala.; Bicine from Sigma, St Louis, Mo.; SUPER-OSETM 6 column from Pharmacia, Piscataway, N.J.; PBS as a 10x concentrate from Life Technologies, Gaithersburg, Md.; Glycerol from J. T. Baker, Phillipsburg, N.J.; and Bis-Tris precast gels from Novex, Calif.

20K PEG strands functionalized with amine-specific ter-15 minal moieties were used in small-scale reaction studies that were set-up to evaluate different reaction conditions in which the PEG:protein stoichiometry was varied. Based on these reactions and the analyses of samples on standard SDS-PAGE, Flt1(1-3)-Fc at a concentration of 1.5 mg/mL was reacted at pH 8.1 with 20K SPA-PEG (PEG succinimidyl propionate) molecules at a PEG-to-Flt1(1-3)-Fc monomer molar ratio of 1:6. The reaction was allowed to proceed at 8° C. overnight. For initial purification, the reaction products were applied to a 10 mm×30 cm SUPEROSE™ 6 column equilibrated with PBS containing 5% Glycerol. The column appeared to separate pegylated Flt1(1-3)-Fc molecules based on the extent of pegylation. Fractions corresponding to what appeared to be primarily mono-pegylated and di-pegylated dimeric Flt1(1-3)-Fc, as judged by banding patterns on reducing and non-reducing SDS-PAGE gels were pooled. The protein concentration was determined by measuring absorbance at 280 nm. The pegylated Flt1(1-3)-Fc protein was sterile filtered, aliquoted and stored at -40° C.

#### Example 6

# Binding of Unmodified, Acetylated, and Pegylated Flt1(1-3)-Fc in a BIACORETM-Based Assay

Unmodified, acetylated, and pegylated Flt1(1-3)-Fc proteins were tested in a BIACORETM-based assay to evaluate their ability to bind to the Flt1 ligand, VEGF. In this assay, unmodified Flt1(1-3)-Fc protein was immobilized on the surface of a BIACORETM chip (see BIACORETM Instruction Manual, Pharmacia, Inc., Piscataway, N.J., for standard procedures) and a sample containing  $0.2 \,\mu\text{g/ml}$  VEGF and either unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc or pegylated Flt1(1-3)-Fc (each at 25  $\mu\text{g/ml})$  was passed over the Flt1(1-3)-Fc-coated chip. To minimize the effects of nonspecific binding, the bound samples were washed with a 0.5 M NaCl wash. In one sample, unmodified Flt1(1-3)-Fc was mixed with heparin. Heparin is a negatively charged molecule and the Flt1(1-3)-Fc protein is a positively charged molecule, so when the two molecules are mixed together, they should interact through their respective charges. This essentially neutralizes Flt1(1-3)-Fc's inherent positive charge making the molecule behave as if it has been chemically or genetically modified so as to reduce its charge and its tendency to bind via charge interactions. As shown in FIG. 3, acetylated (columns 13-16), pegylated (columns 17-20), and heparintreated Flt1(1-3)-Fc (columns 21-24) are each able to completely compete with the BIACORE™ chip-bound Flt1(1-3)-Fc for VEGF binding as compared to control (columns 1-4) and irrelevant protein (columns 5-8). Unmodified Flt1(1-3)-Fc (columns 5-6) appeared to only partially compete with BIACORE™ chip-bound Flt1(1-3)-Fc for VEGF binding. However, washing the bound samples with 0.5 M NaCl (col-

umns 7-8) resulted in a binding profile similar to the modified forms of Flt1(1-3)-Fc, indicating that the unmodified protein was exhibiting non-specific binding to the chip that could be eliminated by the salt wash.

#### Example 7

# Binding of Unmodified, Acetylated, and Pegylated Flt1(1-3)-Fc in an ELISA-Based Assay

Unmodified, acetylated, and pegylated Flt1(1-3)-Fc proteins were tested in a standard ELISA-based assay to evaluate their ability to bind the Flt1 receptor ligand VEGF. As shown in FIG. **4**, both pegylated and acetylated Flt1(1-3)-Fc proteins are capable of binding to VEGF, demonstrating that modify-¹⁵ ing the protein either by pegylation or acetylation does not destroy its ability to bind its ligand.

## Example 8

## Pharmacokinetic Analysis of Unmodified Flt1(1-3)-Fc, Acetylated Flt1(1-3)-Fc, and Pegylated Flt1(1-3)-Fc

In vivo experiments were designed to assess the pharma- ²⁵ cokinetic profiles of unmodified Flt1(1-3)-Fc, acetylated Flt1 (1-3)-Fc, and pegylated Flt1(1-3)-Fc protein. Balb/c mice (23-28 g; 3 mice/group) were injected subcutaneously with 4 mg/kg of unmodified, acetylated, or pegylated Flt1(1-3)-Fc. The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days, and 3 days after injection of protein. The sera were assayed in a standard ELISA-based assay designed to detect Flt1(1-3)-Fc protein. Briefly, the assay involves coating an ELISA plate with VEGF, binding the unmodified, acetylated, or pegylated Flt1(1-3)-Fc-containing sera, and reporting with an anti-Fc ³⁵ antibody linked to alkaline phosphatase. As shown in FIG. 5, the  $T_{max}$  for all of the Flt1(1-3)-Fc proteins was between the 6 hour and 24 hour time points. The  $C_{max}$  for the different proteins was as follows: Unmodified: 0.06µ/ml -0.15 µg/ml; acetylated: 1.5 µg/ml-4.0 µg/ml; and pegylated: approxi- 40 mately 5 µg/ml.

#### Example 9

## Step-Acetylation of Flt1(1-3)-Fc

To determine what minimal amount of acetylation is necessary to eliminate binding to extracellular matrix components, an experiment was designed that acetylated the Flt1(1-3)-Fc protein in a step-wise fashion by using increasing ⁵⁰ amounts of molar excess of acetylation reagent in the acetylation reaction mixture. The range of molar excess was as follows: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 moles of acetylation reagent per 1 mole of Flt1(1-3)-Fc monomer. The reactions were performed as detailed in the instruction ⁵⁵ manual provided with the sulfo-NHS-Acetate modification kit (Pierce Chemical Co., Rockford, Ill., Cat.# 26777).

# Example 10

# Characterization of Step-Acetylated Flt1(1-3)-Fc

IEF analysis Unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc proteins were analyzed by standard IEF analysis. As shown in FIGS. **6A-6**B, unmodified Flt1(1-3)-Fc protein was not able to migrate into the gel due to its extremely high pI (greater than 9.3). However, most of the step-acety-

lated Flt1(1-3)-Fc samples (30-100 fold molar excess samples) were able to migrate into the gel and equilibrate at pIs ranging between 4.55-8.43, depending on the degree of acetylation of the protein. This result demonstrates that acetylation can change the positive charge of the protein in a dose-dependent manner and that reduction of the pI can be controlled by controlling the degree of acetylation.

Binding of Step-acetylated Flt1(1-3)-Fc to Extracellular Matrix Components

To test for binding to extracellular matrix components, Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc where tested in the above-described assay designed to mimic the interaction with extracellular matrix components. Varying concentrations of either unmodified Flt1(1-3)-Fc, step-acetylated Flt1 (1-3)-Fc (10, 20, and 30 fold molar excess samples), or rTie2-Fc (an irrelevant control) protein were added to the wells. The plates were incubated for 1-2 hours at room temperature or 37° C. and then detection of bound proteins was accom-20 plished by adding a secondary alkaline phosphatase-conjugated anti-human Fc antibody to the wells. Alkaline phosphatase substrate was subsequently added to the wells and optical density measured. FIG. 7 shows the results of this assay. Like the irrelevant control protein rTie2-Fc, step-acetylated Flt1(1-3)-Fc (20 and 30 fold molar excess samples) did not exhibit any significant binding to the MATRIGEL® coated plate, whereas the non-acetylated Flt1(1-3)-Fc protein exhibited significant binding. The binding is saturable, indicating that the Flt1(1-3)-Fc protein may be binding to specific sites, rather than a more general charge-mediated interaction that might not be saturable. The 10 fold molar excess sample showed reduced binding, but the degree of acetylation was not enough to completely block binding to extracellular matrix components. The 20 fold molar excess and higher samples displayed no detectable binding, despite the fact that by IEF analysis (FIGS. 6A and 6B) the lower molar excess samples still had a large net positive charge. This result demonstrates that it is not necessary to completely acetylate all available basic amino acids in order to eliminate binding to extracellular matrix components.

Binding of Step-acetylated Flt1(1-3)-Fc in a BIACORETMbased Assay.

Unmodified and step-acetylated Flt1(1-3)-Fc proteins where tested in a BIACORETM-based assay to evaluate their 45 ability to bind to the Flt1 ligand, VEGF. In this assay, unmodified Flt1(1-3)-Fc protein (0.5, 1.0, or 5.0 µg/ml) was immobilized on the surface of a BIACORETM chip (see BIA-CORETM Instruction Manual, Pharmacia, Inc., Piscataway, N.J., for standard procedures) and a solution containing 0.2 µg/ml VEGF and either unmodified Flt1(1-3)-Fc (at either 0.5, 1.0, or 5.0 µg/ml) or 10 different step-acetylated Flt1(1-3)-Fc samples (at 0.5, 1.0, or 5.0 µg/ml each) were passed over the Flt1(1-3)-Fc-coated chip. As shown in FIG. 8, at a sub-stoichiometric ratio (0.5 µg/ml of either unmodified Flt1 (1-3) or step-acetylated Flt1(1-3)-Fc vs. 0.2 µg/ml VEGF), there is not enough Flt1(1-3)-Fc (either unmodified or stepacetylated) in the solution to completely bind the VEGF. At 1.0 µg/ml, which approximates a 1:1 stoichiometric ratio, both unmodified and step-acetylated Flt1(1-3)-Fc are better 60 able to compete for VEGF binding, but there is still insufficient Flt1(1-3)-Fc protein (either unmodified or step-acetylated) to completely bind the available VEGF. However, at 5.0  $\mu$ g/ml, which is several times greater than a 1:1 stoichiometric ratio, both the Flt1(1-3)-Fc and the step-acetylated Flt1(1-3)-Fc proteins are able to bind the VEGF, regardless of the degree of acetylation. This clearly demonstrates that acetylation does not alter Flt1(1-3)-Fc's ability to bind VEGF.

Pharmacokinetic Analysis of Step-acetylated Flt1(1-3)-Fc In vivo experiments were designed to assess the pharmacokinetic profiles of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc protein. Balb/c mice (23-28 g) were injected subcutaneously with 4 mg/kg of unmodified or 10, 20, 40, 60 and 100 fold molar excess samples of step-acetylated Flt1(1-3)-Fc (3 mice for unmodified, 10, 20 and 40 fold molar excess samples and 2 mice for 60 and 100 fold molar excess samples). The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days and 3 days after injection. The sera were assayed in an 10 ELISA-based assay designed to detect Flt1(1-3)-Fc (described supra). FIG. 9 details the results of this study. The T_{max} for all of the Flt1(1-3)-Fc proteins tested was at the 6 hour time point but the  $C_{max}$  was as follows: Unmodified Flt1(1-3)-Fc: 0.06 µg/ml; 10 fold molar excess sample: -0.7 15 µg/ml, 20 fold molar excess sample-2 µg/ml, 40 fold molar excess sample-4 µg/ml, 60 fold molar excess sample-2 µg/ml, 100 fold molar excess sample-1 µg/ml. This results demonstrates that acetylation or pegylation of Flt1(1-3)-Fc significantly improves its pharmacokinetic profile.

## Example 11

# Construction of Flt1(1-3)-Fc Basic Region Deletion Mutant Designated Mut1: Flt1( $1-3_{AB}$ )-Fc

Based on the observation that acetylated Flt1(1-3)-Fc, which has a pI below 6, has much better pharmacokinetics than the highly positive unmodified Flt1(1-3)-Fc (pI>9.3), it was asked whether the difference in pharmacokinetics could 30 be attributed to the net charge of the protein, which made it stick to negatively charged extracellular matrix components, or whether there were perhaps specific locations on the surface of the Flt1(1-3)-Fc protein that constituted specific binding sites for extracellular matrix components. For example, 35 many proteins are known to have heparin binding sites, often consisting of a cluster of basic residues. Sometimes these residues are found in a cluster on the primary sequence of the protein; some of the literature has identified "consensus sequences" for such heparin binding sites (see for example 40 Hileman, et al., 1998, Bioessays 20(2):156-67). In other cases, the known crystal structure of a protein reveals a cluster of positively charged residues on the surface of a protein, but the residues come from different regions of the primary sequence and are only brought together when the protein 45 folds into its tertiary structure. Thus it is difficult to deduce whether an isolated amino acid residue forms part of a cluster of basic residues on the surface of the protein. However, if there is a cluster of positively charged amino acid residues in the primary sequence, it is not unreasonable to surmise that 50 the residues are spatially close to one another and might therefore be part of an extracellular matrix component binding site. Flt1 receptor has been studied extensively and various domains have been described (see for example Tanaka et al., 1997, Jpn. J. Cancer Res. 88:867-876). Referring to the 55 nucleic acid and amino acid sequence set forth in FIGS. 10A-10D of this application, one can identify the signal sequence for secretion which is located at the beginning of the sequence and extends to the glycine coded for by nucleotides 76-78. The mature protein begins with Ser-Lys-Leu-Lys, 60 starting at nucleotide 79 of the nucleic acid sequence. Flt1 Ig domain 1 extends from nucleotide 79 to 393, ending with the amino acids Ser-Asp-Thr. Flt1 Ig domain 2 extends from nucleotide 394 to 687 (encoding Gly-Arg-Pro to Asn-Thr-Ile), and Flt1 Ig domain 3 extends from nucleotides 688 to 996 (encoding Ile-Asp-Val to Asp-Lys-Ala). There is a bridging amino acid sequence, Gly-Pro-Gly, encoded by nucleotides

997-1005, followed by the nucleotide sequence encoding human Fc (nucleotides 1006-1701 or amino acids Glu-Pro-Lys to Pro-Gly-Lys-stop).

A more detailed analysis of the Flt1 amino acid sequence reveals that there is a cluster, namely, amino acid residues 272-281 (KNKRASVRR) of FIGS. 10A-10D, in which 6 out of 10 amino acid residues are basic. This sequence is located in Flt1 Ig domain 3 of the receptor (see FIG. 11), which is not itself essential for binding of VEGF ligand, but which confers a higher affinity binding to ligand. An alignment of the sequence of Ig domain 3 with that of Ig domain 2 reveals that in this region, there is very poor alignment between the two Ig domains, and that there are about 10 additional amino acids in Ig domain 3. An analysis of the hydrophilicity profiles (MACVECTOR[™] computer software) of these two domains clearly indicates the presence of a hydrophilic region in the protein (FIGS. 12A-12B). These observations raised the possibility that the actual three dimensional conformation of Flt1 Ig domain 3 allowed for some type of protrusion that is not in Flt1 Ig domain 2. To test this hypothesis, the additional amino acids were deleted and the resulting protein was tested to see whether the deletion would affect the pharmacokinetics favorably without seriously compromising the affinity of the 25 receptor for VEGF. This DNA construct, which was constructed using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, Mass.), is referred to as Mut1: Flt1(1-3 $_{\Delta B}$ )-Fc. The Mut1: Flt1(1-3 $_{\Delta B}$ )-Fc construct was derived from Flt1(1-3)-Fc by deletion of nucleotides 814-843 (set forth in FIGS. 10A-10D), which deletes the highly basic 10-amino acid residue sequence Lys-Asn-Lys-Arg-Ala-Ser-Val-Arg-Arg-Arg from Flt1 Ig domain 3.

The final DNA construct was sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, Calif.). The sequence of Mut1: Flt1(1-3_{$\Delta B$})-Fc is set forth in FIGS. **13**A-**13**D.

## Example 12

# Construction of Flt1(1-3)-Fc Basic Region Deletion Mutant Designated Mut2: Flt1( $2-3_{\Delta B}$ )-Fc

A second deletion mutant construct, designated Mut2: Flt1  $(2-3_{AB})$ -Fc, was derived from the Mut1: Flt1 $(1-3_{AB})$ -Fc construct by deletion of Flt1 Ig domain 1 encoded by nucleotides 79-393 (see FIGS. 10A-10D); for convenience, nucleotides 73-78 (TCA GGT) were changed to TCC GGA. This introduced a restriction site (BspE1) without altering the associated amino acid sequence, Ser-Gly. This DNA construct, which was constructed using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY)) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, Mass.), was also sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, Calif.). The sequence of Mut2: Flt1(2-3_{$\Delta B$})-Fc is set forth in FIGS. 14A-14C.

# Example 13

# Construction of Flt1(1-3)-Fc Deletion Mutant Designated Mut3: Flt1(2-3)-Fc

A third deletion mutate construct, designated Mut3: Flt1 (2-3)-Fc, was constructed the same way as the Mut2: Flt1(2- $3_{\Delta B}$ )-Fc construct, except that Flt1 Ig domain 3 was left intact (the basic region amino acids were not deleted). The construct was constructed using standard molecular biology techniques ¹⁰ and the final construct was sequence-verified as described supra. The sequence of Mut3: Flt1(2-3)-Fc is set forth in FIGS. **15A-15C**.

#### Example 14

# Construction of Flt1(1-3)-Fc Basic Region N-glycosylation Mutant Designated Mut4: Flt1( $1-3_{R->N}$ )-Fc

A final construct was made in which a N-glycosylation site ²⁰ was introduced into the middle of the basic region of Flt1 Ig domain 3. This construct was designated Mut4: Flt1 (1-3_{*R*->*N*})-Fc and was made by changing nucleotides 824-825 from GA to AC, consequently changing the coded Arg residue (AGA) into an Asn residue (AAC) (see FIGS. **10A-10D**). The ²⁵ resulting amino acid sequence is therefore changed from Arg-Ala-Ser to Asn-Ala-Ser, which matches the canonical signal (Asn-Xxx-Ser/Thr) for the addition of a N-glycosylation site at the Asn residue. The sequence of Mut4: Flt1 (1-3_{*R*->*N*})-Fc is set forth in FIGS. **16A-16**D. 30

#### Example 15

Characterization of Acetylated Flt1(1-3)-Fc, Mut1: Flt1(1-3_{AR})-Fc, and Mut4: Flt1(1-3_{R->N})-Fc Mutants

Binding to Extracellular Matrix Components

To determine whether the three modified proteins were more or less likely to have improved pharmacokinetic properties, MATRIGEL® coated 96-well dishes (as described 40 supra) were incubated with varying concentrations of the mutant proteins and detected with anti-human Fc/alkalinephosphatase conjugated antibodies. As shown in FIG. 18, this experiment showed that while the unmodified Flt1(1-3)-Fc protein could bind avidly to these wells, the Mut3: Flt1(2-3)-45 Fc protein bound somewhat more weakly, the Mut1: Flt1(1- $3_{AB}$ )-Fc protein bound more weakly still, and the Mut2: Flt1  $(2-3_{\Delta B})$ -Fc protein showed the best profile, binding more weakly than any of the other mutant proteins. The Mut4: Flt1  $(1-3_{R->N})$ -Fc glycosylation mutant protein showed only mar- 50 ginal benefit on the MATRIGEL® assay. These results confirm the hypothesis that a linear sequence of positive amino acids can be deleted from the primary sequence resulting in a decrease in charge interaction with extracellular matrix components.

Binding of Mut1: Flt1(1-3_{$\Delta B$})-Fc and Mut4: Flt1(1-3 R_{>N})-Fc in a BIACORETM-based assay.

Unmodified and acetylated Flt1(1-3)-Fc and genetically modified Mut1: Flt1(1-3_{$\Delta B$})-Fc and Mut4: Flt1 (1-3_{R-N})-Fc proteins where tested in a BIACORETM-based assay to evalu- 60 ate their ability to bind to the Flt1 ligand, VEGF. In this assay, unmodified Flt1(1-3)-Fc protein (0.25, 0.5, or 1.0 µg/ml) was immobilized on the surface of a BIACORETM chip (see BIA-CORETM Instruction Manual, Pharmacia, Inc., Piscataway, N.J., for standard procedures) and a solution containing 0.1 65 µg/ml VEGF and either purified or COS cell supernatant containing unmodified Flt1(1-3)-Fc (at approximately (0.25, 0.5).

0.5, or 1.0 µg/ml), purified acetylated Flt1(1-3)-Fc (at (0.25, 0.5, or 1.0 µg/ml), COS cell supernatant containing Mut1: Flt1(1-3_{AB})-Fc (at approximately (0.25, 0.5, or 1.0  $\mu$ g/ml), or COS cell supernatant containing Mut4: Flt1(1-3_{*R*->*N*})-Fc (at approximately (0.25, 0.5, or 1.0 µg/ml) were passed over the Flt1(1-3)-Fc-coated chip. As shown in FIG. 17, at the substoichiometric ratio (0.25 µg/ml Flt1(1-3)-Fc of unmodified, acetylated or genetically modified samples vs. 0.1 µg/ml VEGF), there is insufficient Flt1(1-3)-Fc protein to block binding of VEGF to the Flt1(1-3)-Fc immobilized on the BIACORE™ chip. At 0.5 µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, the stoichiometric ratio approximates 1:1 and there is an increased ability to block VEGF binding to the BIACORE™ chip. At 1.0 µg/ml of ¹⁵ unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, which is approximately a 10:1 stoichiometric ratio, the Flt1(1-3)-Fc proteins are able to block binding of VEGF to the BIACORE™ chip, but they are not equivalent. Unmodified, acetylated, and Mut1:  $Flt1(1-3_{\Delta B})$ -Fc are essentially equal in their ability to block VEGF binding, whereas Mut4: Flt1 (1-3_{*R*->*N*})-Fc is somewhat less efficient at blocking binding. These results confirm the hypothesis that it is possible to reduce the non-specific binding of a positively charged molecule by genetically removing a linear sequence of predominantly negatively charged amino acids.

Binding of Mut1: Flt1(1-3_{$\Delta B$})-Fc, Mut2: Flt1(2-3_{$\Delta B$})-Fc, Mut3: Flt1(2-3)-Fc, and in an ELISA-based assay.

To determine whether the three mutant proteins could bind the Flt1 ligand VEGF, binding experiments were done in which 96-well plates coated with VEGF were incubated with varying concentrations of the respective mutant protein, and after washing, the amount bound was detected by incubating with an alkaline phosphatase conjugated anti-human Fc antibody and quantitated colorimetrically by the addition of an appropriate alkaline phosphatase substrate. As shown in FIG. **19**, this experiment showed that all the mutant proteins could bind VEGF similarly, at the concentrations tested.

#### Example 16

### Pharmacokinetic Analysis of Acetylated Flt1(1-3)-Fc, Mut1: Flt1(1-3_{$\Delta B$})-Fc, and Unmodified Flt1(1-3)-Fc

In vivo experiments were designed to assess the pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1- $3_{\Delta B}$ )-Fc, and 40 fold molar excess acetylated Flt1(1-3)-Fc protein. Balb/c mice (25-30 g) were injected subcutaneously with 4 mg/kg of unmodified Flt1(1-3)-Fc, 40 fold molar excess acetylated Flt1(1-3)-Fc, and Mut1: Flt1(1-3_{\Delta B})-Fc proteins (4 mice each). These mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days, 3 days, and 5 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc protein which involves coating an ELISA plate with VEGF, binding the Flt1(1-3)-Fc and reporting with an anti-Fc antibody linked to alkaline phosphatase. As shown in FIG. **20**, the  $C_{max}$  for these reagents was as follows: Unmodified Flt1(1-3)-Fc-0.15 µg/ml; 40 fold molar excess acetylated Flt1(1-3)-Fc-1.5 µg/ml; and Mut1: Flt1 (1-3_{$\Delta B$})-Fc-0.7 µg/ml.

# Example 17

#### Modified Flt1 Receptor Vector Construction

The rationale for constructing modified versions of the Flt1 receptor (also known as VEGFR1) was based on the observation that the protein sequence of Flt1 was highly basic, and

was therefore likely to stick to extracellular matrix (ECM). The highly basic nature of Flt1 probably explains why unmodified Flt1(1-3)-Fc (described supra) has poor pharmacokinetics that make it difficult to use as a therapeutic agent. As described supra, the chemically modified form of 40 fold ⁵ molar excess acetylated Flt1(1-3)-Fc, hereinafter termed A40, exhibited a greatly improved pharmacokinetic (PK) profile over the non-acetylated Flt1(1-3)-Fc. Therefore, attempts were made to engineer DNA molecules that could be used to recombinantly express modified forms of a Flt1 receptor molecule that would possess the improved PK profile exhibited by A40 and still maintain the ability to bind tightly to VEGF.

It is known in the literature that the first Ig domain of Flt1 15 (which has a net charge of +5 at neutral pH) is not essential for tight binding to VEGF, so this domain was deleted. The third Ig domain (having a net charge of +11) is not essential for binding, but confers higher affinity for VEGF than the second Ig domain, so instead of deleting it entirely, it was replaced 20 with the equivalent domains of the Flt1 receptor relatives Flk1 (also known as VEGFR2) and Flt4 (also known as VEGFR3). These chimeric molecules (denoted R1R2 (Flt1.D2.Flk1D3.Fc\DeltaC1(a) and VEGFR1R2-Fc\DeltaC1(a)) and 25 R1R3 (Flt1 D2.VEGFR3D3-Fc∆C1(a) and VEGFR1R3- $Fc\Delta C1(a)$ ) respectively, wherein R1 and Flt1 D2=Ig domain 2 of Flt1 (VEGFR1); R2 and Flk1D3=Ig domain 3 of Flk1 (VEGFR2); and R3 and VEGFR3D3=Ig domain 3 of Flt4 (VEGFR3)) were much less sticky to ECM, as judged by an 30 in vitro ECM binding assay as described infra, had greatly improved PK as described infra. In addition, these molecules were able to bind VEGF tightly as described infra and block phosphorylation of the native Flk1 receptor expressed in endothelial cells as described infra.

Construction of the Expression Plasmid pFlt1D2.Flk1D3.Fc\DeltaC1(a)

Expression plasmids pMT21.Flt1(1-3).Fc (6519 bp) and pMT21.Flk-1(1-3).Fc (5230 bp) are plasmids that encode ampicillin resistance and Fc-tagged versions of Ig domains 1-3 of human Flt1 and human Flk1, respectively. These plasmids were used to construct a DNA fragment consisting of a fusion of Ig domain 2 of Flt1 with Ig domain 3 of Flk1, using PCR amplification of the respective Ig domains followed by further rounds of PCR to achieve fusion of the two domains into a single fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows:

5': bsp/flt1D2		50
	(SEQ ID NO:18)	
(5'-GACTAGCAGTCCGGAGGTAGACCTTTCGT)	AGAGATG-3')	
3': Flt1D2-Flk1D3.as		
	(SEQ ID NO:19)	
(5'-CGGACTCAGAACCACATCTATGATTGTAT	TGGT-3')	55

The 5' amplification primer encodes a BspE1 restriction enzyme site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (SEQ ID NO:20) (corresponding to amino acids 27-33 of FIGS. **21A-21**C). The 3' 60 primer encodes the reverse complement of the 3' end of Flt1 Ig domain 2 fused directly to the 5' beginning of Flk1 Ig domain 3, with the fusion point defined as TIID (SEQ ID NO:37) of Flt1 (corresponding to amino acids 123-126 of FIGS. **21A-21**C) and continuing into VVLS (SEQ ID NO:38) 65 (corresponding to amino acids 127-130 of FIGS. **21A-21**C) of Flk1.

For Ig domain 3 of Flk1, the 5' and 3' amplification primers were as follows:

```
5': Flt1D2-Flk1D3.s
```

```
(SEQ ID NO:21) (5'-ACAATCATAGATGTGGTTCTGAGTCCGTCTCATGG-3')
```

The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused directly to the beginning of Flk1 Ig domain 3, as described above. The 3' amplification primer encodes the end of Flk1 Ig domain 3, defined by the amino acids VRVHEK (SEQ ID NO:23) (corresponding to amino acids 223-228 of FIGS. **21A-21**C), followed by a bridging sequence that includes a recognition sequence for the restriction enzyme Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 229-231 of FIGS. **21A-21**C.

After a round of PCR amplification to produce the individual domains, the products were combined in a tube and subjected to a further round of PCR with the primers bsp/ flt1D2 and Flk1D3/apa/srf.as (described supra) to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 614 bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/ $\Delta$ B2.Fc, to create the plasmid pMT211Flt1D2.Flk1D3.Fc. The nucleotide sequence of the Flt1D2-Flk1D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 702 bp fragment was transferred into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)B2-Fc $\Delta$ C1(a) to produce the plasmid pFlt1 D2. Flk1D3.Fc $\Delta$ C1(a). The com-35 plete DNA and deduced amino acid sequences of the Flt1D2.Flk1D3.Fc $\Delta$ C1(a) chimeric molecule is set forth in FIGS. 21A-21C.

Construction of the Expression Plasmid pFlt1D2VEGFR3D3FCAC1(a)

The expression plasmid pMT21.Flt1(1-3).Fc (6519 bp) encodes ampicillin resistance and an Fc-tagged version of Ig domains 1-3 of human Flt1 receptor. This plasmid was used to produce a DNA fragment containing Ig domain 2 of Flt1 by PCR. RNA from the cell line HEL921.7 was used to produce Ig domain 3 of Flk1, using standard RT-PCR methodology. A further round of PCR amplification was used to achieve fusion of the two Ig domains into a single fused fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows:

5': bsp/flt1D2

(SEQ ID NO :24) (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3')

3': Flt1D2.VEGFR3D3.as

(SEQ ID NO:25) (TTCCTGGGCAACAGCTGGATATCTATGATTGTATTGGT)

The 5' amplification primer encodes a BspE1 restriction site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (SEQ ID NO:20) (corresponding to amino acids 27-33 of FIGS. **22A-22**C). The 3' amplification primer encodes the reverse complement of the end of Flt1 Ig domain 2 fused directly to the beginning of VEGFR3 Ig domain 3, with the fusion point defined as TIID (SEQ ID NO:37) of Flt1 (corresponding to amino acids 123-126 of FIGS. **22A-22**C) and continuing into IQLL (SEQ ID NO:26) of VEGFR3 (corresponding to amino acids 127-130 of FIGS. **22A-22**C).

For Ig domain 3 of VEGFR3, the 5' and 3' primers used for RT-PCR were as follows:

5': R3D3.s (SEQ ID NO:27) (ATCCAGCTGTTGCCCAGGAAGTCGCTGGAGCTGCTGGTA)

3': R3D3.as

(SEQ ID NO: 28) (ATTTTCATGCACAATGACCTCGGTGCTCTCCCGAAATCG)

Both the 5' and 3' amplification primers match the sequence of VEGFR3. The 296 bp amplification product of this RT-PCR reaction was isolated by standard techniques and subjected to a second round of PCR to add suitable sequences to 15 allow for fusion of the Flt1D2 with the Flk1D3 domains and fusion of the Flk1D3 and Fc domains via a GPG bridge (see below). The amplification primers were as follows:

5': Flt1D2.VEGFR3D3.s (SEO ID NO:29) (TCATAGATATCCAGCTGTTGCCCAGGAAGTCGCTGGAG) 3': VEGFR3D3/srf.as

(SEQ ID NO:30) (GATAATGCCCGGGCCATTTTCATGCACAATGACCTCGGT)

The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' end of VEGFR3 Ig domain 3, defined by the 30 amino acids VIVHEN (SEQ ID NO:31) (corresponding to amino acids 221-226 of FIGS. 22A-22C), followed by a bridging sequence that includes a recognition sequence for Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 227-229 of FIGS. 22A- 35 **22**C

After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain 3) of PCR to produce the individual Ig domains, the PCR products were combined in a tube and subjected to a further round of PCR amplification with the 40 amplification primers bsp/flt1D2 and VEGFR3D3/srf.as described supra, to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 625 bp fragment was subcloned into the BspEI to SrfI restriction sites of the 45 vector pMT21/Flt1ΔB2.Fc (described supra), to create the plasmid pMT21/Flt1D2.VEGFR3D3.Fc. The sequence of the Flt1D2-VEGFR3D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 50 693 bp fragment was subcloned into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3) $\Delta$ B2-Fc $\Delta$ C1(a) to produce the plasmid designated pFlt1 D2.VEGFR3D3.Fc $\Delta$ C1(a). The complete DNA deduced amino acid sequence of the Flt1 D2.VEGFR3D3.Fc\DeltaC1(a) chimeric molecule is set forth in 55 FIGS. 22A-22C.

#### Example 18

Extracellular Matrix Binding (ECM) Binding Assay

ECM-coated plates (Becton Dickinson catalog #35-4607) were rehydrated with warm DME supplemented with glutamine (2 mM), 100 U penicillin, 100 U streptomycin, and 10% BCS for at least 1 hr before adding samples. The plates 65 were then incubated for 1 hr at room temperature with varying Flt1D2.Flk1D3.Fc $\Delta$ C1(a) concentrations of and

Flt1D2.VEGFR3D3.Fc\DeltaC1(a) starting at 10 nM with subsequent 2-fold dilutions in PBS plus 10% BCS. The plates were then washed 3 times with PBS plus 0.1% Triton-X and incubated with alkaline phosphatase-conjugated anti-human Fc antibody (Promega, 1:4000 in PBS plus 10% BCS) for 1 hr at room temperature. The plates were then washed 4 times with PBS 0.1% Triton-X and alkaline phosphatase buffer/pNPP solution (Sigma) was added for color development. Plates were read at I=405-570 nm. The results of this experiment are ¹⁰ shown in FIG. 23 and demonstrate that the  $Flt1D2.Flk1D3.Fc\Delta C1(a) \ and \ Flt1D2.VEGFR3D3.Fc\Delta C1$ (a) proteins are considerably less sticky to the ECM as compared to the Flt1(1-3)-Fc protein.

#### Example 19

# Transient Expression of pFlt1D2.Flk1D3.FcΔC1(a) in CHO-K1 (E1A) Cells

A large scale (2 L) culture of E. coli DH10B cells carrying the pFlt1D2.Flk1D3.Fc∆C1(a) plasmid described supra in Example 17 was grown overnight in Terrific Broth (TB) plus 100 µg/ml ampicillin. The next day, the plasmid DNA was extracted using a QIAgen ENDOFREE™ Megaprep kit fol-25 lowing the manufacturer's protocol. The concentration of the purified plasmid DNA was determined by standard techniques using a UV spectrophotometer and fluorometer. The plasmid DNA was verified by standard restriction enzyme digestion of aliquots using the restriction enzymes EcoRI plus NotI and AseI. All restriction enzyme digest fragments corresponded to the predicted sizes when analyzed on a 1% agarose gel.

Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a density of 4×10⁶ cells/plate. Plating media was Gibco Ham's F-12 supplemented with 10% HYCLONE™ Fetal Bovine Serum (FBS), 100 U penicillin/100 U streptomycin and glutamine (2 mM). The following day each plate of cells was transfected with  $6 \mu g$  of the pFlt1D2.Flk1D3.Fc $\Delta$ C1 (a) plasmid DNA using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following the manufacturer's protocol. Four hours after adding the transfection mix to the cells, 12 ml/plate of Optimem supplemented with 10% FBS was added. Plates were incubated at 37° C. in a 5% CO₂ incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II supplemented with glutamine (2 mM) and 1 mM sodium butyrate) was added. The plates were incubated at 37° C. for 3 days. After 3 days of incubation, the media was aspirated from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet cells. The supernatant was decanted into sterile 1 L bottles and purification of the expressed protein was performed as described infra.

# Example 20

## Construction pVEGFR1R2-Fc∆C1(a) Expression Vector

The pVEGFR1R2.Fc $\Delta$ C1(a) expression plasmid was con-60 structed by insertion of DNA encoding amino acids SDT (corresponding to amino acids 27-29 of FIGS. 24A-24C) between Flt1d2-Flk1d3-Fc∆C1(a) amino acids 26 and 27 of FIGS. 21A-21C (GG) and removal of DNA encoding amino acids GPG corresponding to amino acids 229-231 of Figure. The SDT amino acid sequence is native to the Flt1 receptor and was added back in to decrease the likelihood of heterogeneous N-terminal processing. The GPG (bridging

sequence) was removed so that the Flt1 and Flk1 Ig domains were fused directly to one another. The complete DNA and deduced amino acid sequences of the pVEGFR1R2.Fc∆C1 (a) chimeric molecule is set forth in FIGS. 24A-24C.

## Example 21

# Cell Culture Process Used to Produce Modified Flt1 Receptors

Cell Culture Process Used to Produce  $Flt1D2.Flk1D3.Fc\Delta C1(a)$ 

The process for production of Flt1D2.Flk1D3.Fc $\Delta$ C1(a) protein using the expression plasmid pFlt1D2.Flk1D3.Fc∆C1(a) described supra in Example 1 involves suspension culture of recombinant Chinese hamster ovary (CHO K1/E1A) cells which constitutively express the protein product. The cells are grown in bioreactors and the protein product is isolated and purified by affinity and size exclusion chromatography. The process is provided in greater 20 detail below.

Cell Expansion

Two confluent T-225 cm² flasks containing the Flt1D2.Flk1D3.Fc\DeltaC1(a) expressing cell line were expanded by passaging cells into eight T-225 cm² flasks in medium ²⁵ (GMEM+10% serum, GIBCO) and incubated at 37° C. and 5% CO₂. When the flasks approached confluence (approximately 3 to 4 days) the cells were detached using trypsin. Fresh medium was added to protect the cells from further exposure to the trypsin. The cells were centrifuged and resus- 30 pended in fresh medium then transferred to eight 850 cm² roller bottles and incubated at 37° C. and 5% CO2 until confluent.

Suspension Culture in Bioreactors

them from the surface and washed with suspension culture medium. The cells are aseptically transferred to a 5 L bioreactor (New Brunswick Celligen Plus) where the cells are grown in 3.5 L of suspension culture. The suspension culture medium was a glutamine-free low glucose modification of 40 IS-CHO (Irvine Scientific) to which 5% fetal bovine serum (HYCLONE™), GS supplement (Life Technologies) and 25 µM methionine sulfoximine (Sigma) was added. The pH was controlled at 7.2 by addition of carbon dioxide to the inlet gas or by addition of a liquid solution of sodium carbonate to the 45 bioreactor. Dissolved oxygen level was maintained at 30% of saturation by addition of oxygen or nitrogen to the inlet gas and temperature controlled at  $37^{\circ}$  C. When a density of  $4 \times 10^{\circ}$ cells/mL was reached the cells were transferred to a 40 L  $\,$ bioreactor containing the same medium and setpoints for 50 controlling the bioreactor. The temperature setpoint was reduced to 34° C. to slow cell growth and increase the relative rate of protein expression.

Cell	Cul	ture	Process	U	Jsed	to	Proc	luce	
Flt1D2	.VEGFI	R3D3.F	c∆C1(a)						5
The	same	metho	dologies	as	describ	ed	supra	for	

Flt1D2.Flk1D3.Fc $\Delta$ C1(a) were produce used to Flt1D2.VEGFR3D3.FcΔC1(a).

#### Example 22

Harvest and Purification of Modified Flt1 Receptors

Harvest and Purification of Flt1D2.Flk1D3.FcΔC1(a)

The product protein was aseptically harvested from the 65 bioreactor while retaining cells using Millipore Prostak tangential-flow filtration modules and a low-shear mechanical

pump (Fristam). Fresh medium was added to the bioreactor to replace that removed during the harvest filtration. Approximately 40 L of harvest filtrate was then loaded onto a 400 mL column containing Protein A SEPHAROSE™ resin (Amersham Pharmacia). After loading the resin was washed with buffer containing 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.2 to remove any unbound contaminating proteins. Flt1D2.Flk1D3.Fc $\Delta$ C1(a) protein was eluted with a pH 3.0 citrate buffer. The eluted protein was neutralized by addi-¹⁰ tion of Tris base and frozen at  $-20^{\circ}$  C.

Several frozen lots of Flt1D2.Flk1D3.Fc\DeltaC1(a) protein from the Protein A step above were thawed, pooled and concentrated using a Millipore 30 kD nominal molecular weight cutoff (NMWCO) tangential flow filtration membrane. The protein was transferred to a stirred cell concentrator (Millipore) and further concentrated to 30 mg/mL using a 30 kD NMWCO membrane. The concentrated protein was loaded onto a size exclusion column packed with SUPERDEXTM 200 resin (Amersham Pharmacia) that was equilibrated with phosphate buffered saline plus 5% glycerol. The same buffer was used to run the column. The fractions corresponding to Flt1 D2.Flk1D3.FcΔC1(a) dimer were pooled, sterile filtered through a 0.22 micron filter, aliquoted and frozen.

Harvest and Purification of Flt1D2.VEGFR3D3.FcΔC1(a) The same methodologies as described supra for Flt1D2.Flk1D3.Fc $\Delta$ C1(a) were used to harvest and purify Flt1 D2.VEGFR3D3.FcΔC1(a).

#### Example 23

#### Phosphorylation Assay for Transiently Expressed VEGFR2

Primary human umbilical vein endothelial cells (HU-Cells grown in roller bottles were trypsinized to detach 35 VECs), passage 4-6, were starved for 2 hrs in serum-free DME high glucose media. Samples containing 40 ng/ml (1 nM) human VEGF165, which is a ligand for the VEGF receptors Flt1, Flk1 and Flt4(VEGFR3) were prepared and were preincubated for 1 hr at room temperature with varying amounts of the modified Flt1 receptors Flt1(1-3)-Fc, Flt1(1-(A40),  $Flt1D2Flk1D3.Fc\Delta C1(a)$ 3)-Fc and Flt1D2VEGFR3D3.Fc∆C1(a) in serum-free DME-high glucose media containing 0.1% BSA. Cells were challenged for 5 minutes with the samples prepared above +/-VEGF165, followed by whole cell lysis using complete lysis buffer. Cell lysates were immunoprecipitated with an antibody directed against the C-terminus of VEGFR2 receptor. The immunoprecipitated lysates were loaded onto 4-12% SDS-PAGE Novex gel and then transferred to PVDF membrane using standard transfer methodologies. Detection of phosphorylated VEGFR2 was done by immunoblotting with the antiphospho Tyrosine mAb called 4G10 (UBI) and developed using ECL-reagent (Amersham). FIGS. 25A-25C and 26A-26B show the results of this experiment. FIGS. 25A-25C reveals that detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are phosphorylated to varying levels depending on which modified Flt1 receptor is used during the preincubations with VEGF. As is seen in FIG. 25A, at a 1.5 60 molar excess of either Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3.Fc\DeltaC1(a) there is complete blockage of receptor stimulation by these three modified Flt1 receptors as compared to control media challenge. In contrast, transient Flt1D2VEGFR3D3.Fc∆C1(a) does not show significant blockage at this molar excess, as compared with VEGF positive control challenge. Similar results are seen in FIG. 25B, where the modified Flt receptors are in a 3-fold molar excess to VEGF165 ligand. In FIG. 25C, where the modified Flt1 receptors are in a 6-fold molar excess to VEGF165 ligand, transient Flt1D2VEGFR3D3.FcΔC1(a) can now be shown to be partially blocking VEGF165-induced stimulation of cell-surface receptors.

In FIGS. 26A-26B, detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are not phosphorylated by challenge samples which have VEGF165 preincubated with 1 and 2 fold molar excess (FIG. 26A) or 3 and 4¹⁰ fold molar excess (FIG. 26B) of either transient Flt1 D2Flk1D3.Fc\DeltaC1(a), stable Flt1 D2Flk1D3.Fc\DeltaC1(a), or transient VEGFR1R2-Fc $\Delta$ C1(a). At all modified Flt1 receptor concentrations tested there is complete binding of VEGF165 ligand during the preincubation, resulting in no¹⁵ detectable stimulation of cell-surface receptors by unbound VEGF165 as compared to control media challenge.

#### Example 24

# Cell Proliferation Bioassay

The test cell population is MG87 cells that have been stably transfected with a expression plasmid that contains a DNA insert encoding the VEGFR2(Flk1) extracellular domain 25 Fc\DeltaC1(a) at a concentration of 1 nM (estimated to be 1000 fused to the TrkB intracellular kinase domain, thus producing a chimeric molecule. The reason the TrkB intracellular kinase domain was used rather than the native VEGFR2(Flk1) intracellular kinase domain is that the intracellular kinase domain of VEGFR2(Flk1) does not cause a strong proliferative 30 response when stimulated by VEGF165 in these cells. It is known that MG87 cells containing full length TrkB receptor give a robust proliferative response when stimulated with BDNF, so the TrkB intracellular kinase domain was engineered to replace the intracellular kinase domain of VEGFR2 35 (Flk1) to take advantage of this proliferative response capability.

Five thousand cells/well were plated in a 96 well plate and allowed to settle for 2 hrs at 37° C.

The following modified Flt receptors Flt1(1-3)-Fc, 40 Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1 (a), plus an irrelevant receptor termed Tie2-Fc as a negative control, were titrated from 40 nM to 20 µM and incubated on the cells for 1 hr at 37° C. Human recombinant VEGF165 in defined media was then added to all the wells at a concentra- 45 tion of 1.56 nM. The plates were incubated for 72 hrs at 37° C. and then MTS (Owen's reagent, Promega) added and the plates were incubated for an additional for 4 hrs. Finally, the plates were read on a spectrophotometer at 450/570 nm. The results of this experiment are shown in FIG. 27. The control 50 receptor Tie2-Fc does not block VEGF165-induced cell proliferation concentration at any whereas Flt1D2.Flk1D3.Fc\DeltaC1(a) blocks 1.56 nM VEGF165 with a half maximal dose of 0.8 nM. Flt1(1-3)-Fc and Flt1D2.VEGFR3D3.Fc\DeltaC1(a) are less effective in blocking 55 VEGF165 in this assay with a half maximal dose of -2 nM. VEGF165 alone gives a reading of 1.2 absorbance units and the background is 0.38 absorbance units.

#### Example 25

# Binding Stoichiometry of Modified Flt Receptors to VEGF165

# BIACORE™ Analysis

The stoichiometry of Flt1D2Flk1D3.Fc∆C1(a) and VEGFR1R2-Fc∆C1(a) interaction with human VEGF165 was determined by measuring either the level of VEGF saturation binding to the Flt1D2Flk1D3.Fc∆C1(a) or VEGFR1R2-Fc∆C1(a) surfaces or measuring concentration of VEGF165 needed to completely prevent binding of Flt1D2Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a) to VEGF BIACORE™ chip surface.

Modified Flt receptors Flt1D2Flk1D3.Fc\DeltaC1(a) and VEGFR1R2-Fc $\Delta$ C1(a), were captured with an anti-Fc specific antibody that was first immobilized on a BIACORE™ chip using amine-coupling chemistry. A blank antibody surface was used as a negative control. VEGF165 was injected at a concentration of 1 nM, 10 nM, and 50 nM over the Flt1D2Flk1D3.Fc $\Delta$ C1(a) and VEGFR1R2-Fc $\Delta$ C1(a) surfaces at 10 µl/min for one hour. A real-time binding signal was recorded and saturation binding was achieved at the end of each injection. Binding stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1 D2Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a), using the 20 conversion factor of 1000 RU equivalent to 1 ng/ml. The results indicated binding stoichiometry of one VEGF165 dimeric molecule per one Flt1D2Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a) molecule (FIG. 28).

In solution, Flt1D2Flk1D3.Fc\DeltaC1(a) or VEGFR1R2times higher than the KD of the Flt1D2Flk1D3.Fc\DeltaC1(a) or VEGFR1R2-Fc∆C1(a)/VEGF165 interaction) were mixed with varied concentrations of VEGF165. After one hour incubation, concentrations of the free Flt1D2Flk1D3.Fc $\Delta$ C1(a) in solution were measured as a binding signal to an aminecoupled VEGF165 surface. A calibration curve was used to convert the Flt1D2Flk1D3.Fc∆C1(a) BIACORE[™] binding signal to its molar concentration. The data showed that the addition of 1 nMVEGF165 into the Flt1D2Flk1D3.Fc $\Delta$ C1(a) solution completely blocked Flt1D2Flk1D3.Fc∆C1(a) binding to the VEGF165 surface. This result suggested the binding stoichiometry of one VEGF165 molecule per one Flt1D2Flk1D3.Fc $\Delta$ C1(a) molecule (FIG. 29 and FIG. 30). When the concentration of Flt1D2Flk1D3.Fc\DeltaC1(a) was plotted as a function of added concentration of VEGF165, the slope of the linear portion was -1.06 for Flt1D2Flk1D3.Fc $\Delta$ C1(a) and -1,07 for VEGFR1R2-Fc $\Delta$ C1 (a). The magnitude of the slope, very close to negative one, was indicative that one molecule of VEGF165 bound to one molecule of either Flt1D2Flk1D3.Fc\DeltaC1(a) or VEGFR1R2- $Fc\Delta C1(a)$ .

Size Exclusion Chromatography

Flt1D2Flk1D3.Fc $\Delta$ C1(a) was mixed with a 3-fold excess of VEGF165 and the receptor-ligand complex was purified using a Pharmacia SUPEROSETM 6 size exclusion chromatography column. The receptor-ligand complex was then incubated in a buffer containing 6 M guanidine hydrochloride in order to dissociate it into its component proteins. Flt1D2Flk1D3.Fc\DeltaC1(a) was separated from VEGF165 using SUPEROSE™ 6 size exclusion chromatography column run in 6 M guanidium chloride. In order to determine complex stoichiometry, several injections Flt1D2Flk1D3.Fc $\Delta$ C1(a) and VEGF165 were made and peak height or peak integrated intensity was plotted as a function of 60 the concentration of injected protein. The calibration was done under condition identical to one used in separating components of Flt1D2Flk1D3.Fc\DeltaC1(a)/VEGF complex. Quantification of the Flt1D2Flk1D3.Fc∆C1(a)/VEGF complex composition was based on the calibration curves. The results of this experiment are set forth in FIG. 28, which shows the ratio of VEGF165 to Flt1D2Flk1D3.Fc∆C1(a) in a complex to be 1:1.
#### Example 26

### Determination of the Binding Stoichiometry of Flt1D2Flk1D3.FcΔC1(a)/VEGF165 Complex by Size Exclusion Chromatography

Flt1D2Flk1D3.FcΔC1(a)/VEGF165 Complex Preparation VEGF165 (concentration=3.61 mg/ml) was mixed with CHO cell transiently expressed Flt1 D2.Flk1D3.FcΔC1(a) (concentration=0.9 mg/ml) in molar ratio of 3:1 (VEGF165: ¹⁰ Flt1D2.Flk1D3. FcΔC1(a)) and incubated overnight at 4° C.

Size Exclusion Chromatography (SEC) Under Native Conditions

To separate the complex from excess of unbound ¹⁵ VEGF165, 50  $\mu$ l of the complex was loaded on a Pharmacia SUPEROSETM 12 PC 3.2/30 which was equilibrated in PBS buffer. The sample was eluted with the same buffer at flow rate 40  $\mu$ l/min. at room temperature. The results of this SEC are shown in FIG. **31**. Peak #1 represents the complex and ²⁰ peak #2 represents unbound VEGF165. Fractions eluted between 1.1 and 1.2 ml were combined and guanidinium hydrochloride (GuHCl) was added to a final concentration 4.5 M to dissociate the complex.

Size Exclusion Chromatography (SEC) Under Dissocia-  25  tive Conditions

To separate the components of the receptor-ligand complex and to determine their molar ratio, 50  $\mu$ l of dissociated complex as described supra was loaded onto a SUPEROSETM 12 PC 3.2/30 equilibrated in 6 M GuHCl and eluted with the same solution at a flow rate 40  $\mu$ l/min at room temperature. The results of this SEC are shown in FIG. **32**. Peak #1 represents Flt1D2Flk1D3.Fc $\Delta$ C1(a) and peak #2 represents VEGF165.

Calculation of Flt1 D2Flk1D3.Fe $\Delta$ C1(a):VEGF165 Complex Stoichiometry

The stoichiometry of the receptor-ligand complex was determined from the peak area or the peak height of the components. Concentrations of VEGF165 and 40 Flt1D2Flk1D3.Fc $\Delta$ C1(a) corresponding to the peak height or peak area, respectively, were obtained from the standard curves for VEGF165 and Flt1D2Flk1D3.Fc\DeltaC1(a). To obtain a standard curve, four different concentrations (0.04 mg/ml-0.3 mg/ml) of either component were injected onto a Phar- 45 macia SUPEROSE™ 12 PC 3.2/30 column equilibrated in 6 M guanidinium chloride and eluted with the same solution at flow rate 40 µl/min at room temperature. The standard curve was obtained by plotting peak area or peak height vs protein concentration. The molar ratio of VEGF165: 50 Flt1D2Flk1D3.Fc $\Delta$ C1(a) determined from the peak area of the components was 1.16. The molar ratio of VEGF165: Flt1D2Flk1D3.Fc\DeltaC1(a) determined from the peak height of the components was 1.10.

#### Example 27

# Determination of the Stoichiometry of the Flt1D2Flk1D3.Fc $\Delta$ C1(a)/VEGF165 Complex by Size Exclusion Chromatography with On-Line Light Scattering

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#### **Complex Preparation**

VEGF165 was mixed with CHO transiently expressed Flt1D2.Flk1D3.Fc $\Delta$ C1(a) protein in molar ratio of 3:1 65 (VEGF165:Flt1 D2Flk1D3.Fc $\Delta$ C1(a)) and incubated overnight at 4° C.

Size Exclusion Chromatography (SEC) with On-Line Light Scattering

Size exclusion chromatography column with a MiniDawn on-line light scattering detector (Wyatt Technology, Santa Barbara, Calif.) and refractive index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine the molecular weight (MW) of the receptor-ligand complex. Samples were injected onto a SUPEROSE™ 12 HR 10/30 column (Pharmacia) equilibrated in PBS buffer and eluted with the same buffer at flow rate 0.5 ml/min. at room temperature. As shown in FIG. 33, the elution profile shows two peaks. Peak #1 represents the receptor-ligand complex and peak #2 represents the unbound VEGF165. MW was calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.Fc $\Delta$ C1(a)/VEGF165 complex at the peak position is 157 300 (FIG. 33), the MW of VEGF165 at the peak position is 44 390 (FIG. 34) and the MW of R1R2 at the peak is 113 300 (FIG. 35).

These data indicated that the stoichiometry of the Flt1D2Flk1D3.  $Fc\Delta C1(a)/VEGF$  complex is 1:1 as its corresponds to the sum of molecular weights for Flt1D2Flk1D3.  $Fc\Delta C1(a)$  and VEGF165. Importantly, this method conclusively proved that the Flt1 D2Flk1D3. $Fc\Delta C1(a)/VEGF165$  complex was indeed composed of only one molecule of VEGF165 ligand and only one molecule of the Flt1D2Flk1D3. $Fc\Delta C1(a)$ .

#### Example 28

#### Peptide Mapping of Flt1D2.Flk1D3.FcΔC1(a)

The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.Fc $\Delta$ C1(a) were determined by a peptide mapping method. In this method, the protein was first cleaved with trypsin. Tryptic fragments were analyzed and identified by HPLC coupled with mass spectrometry, in addition to an N-terminal sequencing technique. Reduction of the tryptic digest was employed to help identify disulfide-bond-containing fragments. Treatment of the tryptic digest with PNGase F (Glyko, Novato, Calif.) was employed to help identify fragments with N-linked glycosylation sites. The results are summarized in the accompanying FIG. **36**.

There are a total of ten cysteines in Flt1 D2.Flk1D3.Fc∆C1
(a); six of them belong to the Fc region. Cys27 has been confirmed to be disulfide bonded to Cys76. Cys121 is confirmed to be disulfide bonded to Cys182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two cysteines in another Fc chain. However, because these two cysteines can not be separated enzymatically from each other, it can not be
determined whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is confirmed to be disulfide bonded to Cys306. Cys352 is confirmed to be disulfide bonded to Cys410.

There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.Fc $\Delta$ C1(a). All five of them are found to be glycosylated to varying degrees. Complete glycosylation was observed at Asn33 (amino acid sequence NIT), Asn193 (amino acid sequence NST), and Asn282 (amino acid sequence NST). In addition, partial glycosylation is observed on Asn65 and Asn120. Sites of glycosylation are highlighted by underline in the FIG. **36**.

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#### Example 29

Pharmacokinetic Analysis of Modified Flt Receptors

Pharmacokinetic analysis of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and VEGFR1R2-Fc $\Delta$ C1(a)

Balb/c mice (25-30 g) were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1 expressed D2.Flk1D3.Fc $\Delta$ C1(a), CHO stably Flt1D2.Flk1D3.FcΔC1(a), and CHO transiently expressed 10 VEGFR1R2-Fc $\Delta$ C1(a). The mice were tail bled at 1, 2, 4, 6, 24 hrs, 2 days, 3 days and 6 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a). The ELISA involves coating an ELISA plate with VEGF165, Flt1(1-3)-Fc binding the detect (A40). Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. The results of this experiments are shown in FIG. 37. The  $T_{max}$  for Flt1(1-3)-Fc (A40) was at 6 hrs while the 20  $T_{max}$  for the transient and stable Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and the transient VEGFR1R2-Fc $\Delta$ C1(a) was 24 hrs. The C_{max} for Flt1(1-3)-Fc (A40) was 8 µg/ml. For both transients (Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and VEGFR1R2-Fc $\Delta$ C1(a)) the  $C_{max}$  was 18 µg/ml and the  $C_{max}$  for the stable VEGFR1R2- ²⁵ Fc $\Delta$ C1(a) was 30 µg/ml. (b) Pharmacokinetic analysis of  $Flt1D2.Flk1D3.Fc\DeltaC1(a)$ Flt1(1-3)-Fc (A40), and Flt1D2.VEGFR3D3.FcΔC1(a)

Balb/c mice (25-30 g) were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed 30 Flt1D2.Flk1D3.Fc\DeltaC1(a) and CHO transiently expressed Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a). The mice were tail bled at 1, 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc, Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and Flt1D2.VEGFR3D3.Fc $\Delta$ C1 (a). The ELISA involves coating an ELISA plate with VEGF 165, binding the Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) or Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. Flt1(1-3)-Fc (A40) could no longer be detected in the serum after day 5 40 whereas, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a) were detectable for 15 days or more. The results of this experiment are shown in FIG. 38.

### Example 30

#### Evaluation of the Ability of Flt1D2.Flk1D3.FcΔC1(a) to Inhibit Tumor Growth In Vivo

To evaluate the ability of Flt1D2.Flk1D3.Fc∆C1(a) to inhibit tumor growth in vivo a model in which tumor cell suspensions are implanted subcutaneously on the right flank of male severe combined immunodeficiency (SCID) mice was employed. Two cell lines, the human HT-1080 fibrosar- 55 coma cell line (ATCC accession no. CCL-121) and the rat C6 glioma cell line (ATCC accession no. CCL-107), each of which exhibit distinctly different morphologies and growth characteristics, were used in the assay. The first dose of Flt1 D2.Flk1D3.Fc $\Delta$ C1(a) (at 25 mg/Kg or as indicated in FIGS. 60 39 and 40) was given on the day of tumor implantation. Animals subsequently received subcutaneous injections of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or vehicle either every other day (EOD) or two times per week (2×/wk) for a period of 2 weeks. After 2 weeks, animals were perfused 65 with fixative, tumors were removed and samples were blinded. Tumor volume was determined by measuring the

length and width of visible subcutaneous tumors. Both of Flt1(1-3)-Fc (A40) and Flt1D2.Flk1D3.Fc $\Delta$ C1(a) significantly reduced the growth of tumors formed by HT-1080 and C6 cells. The results of these experiments are shown in FIG. **39** and FIG. **40**.

#### Example 31

#### The Effect of VEGF165 and Modified Flt Receptors in Female Reproductive System

The stereotypic pattern of vascular remodeling which occur in the uterus and ovary over the course of the reproductive cycle has been well characterized, making these tissues particularly well suited to the study of mechanisms which regulate angiogenesis, vascular remodeling and vascular regression. Indeed, in situ hybridization studies in the reproductive tissues provided the first clear evidence that VEGF acts as a mediator of physiological angiogenesis in mature rodents, as well as humans and non-human primates (Phillips et al., 1990; Ravindranath et al., 1992; Shweiki et al., 1993; Kamat et al., 1995). As cyclic angiogenesis and vascular remodeling are prominent features of the normal ovary and uterus, it is not surprising that abnormal blood vessel growth and/or vascular dysfunction have been found to characterize many pathological conditions which affect these organs. Furthermore, these pathogenic vascular abnormalities are thought to be caused or perpetuated by the dysregulated expression of one or more angiogenic or anti-angiogenic factors, most prominently VEGF.

For example, abnormal angiogenesis is characteristic of polycystic ovary disease, endometriosis and endometrial carcinoma, and in each case VEGF is over expressed in the affected tissue (Kamat et al., 1995; Shifren et al., 1996; Guidi
et al., 1996; Donnez et al., 1998). Overexpression of VEGF is also thought to play a pathogenic role in the establishment of systemic vascular hyperpermeability in ovarian hyperstimulation syndrome (McClure et al., 1994; Levin et al., 1998) and preeclampsia (Baker et al., 1995; Sharkey et al., 1998). In addition, VEGF has been implicated as the permeability factor responsible for the production of ascites associated with ovarian carcinoma and other tumors (Senger et al., 1983; Boocock et al., 1995). Agents which effectively neutralize the biological actions of VEGF can reasonably be anticipated to 45 be of therapeutic benefit in the above and related conditions.

Angiogenesis and vascular remodeling are also hallmarks of blastocyst implantation and placental development (Findlay, 1986). VEGF is strongly expressed both in the maternal decidua and in embryonic trophoblasts, where it is thought to first stimulate expansion and hyperpermeability of the uterine vasculature during the peri-implantation period and subsequently mediate formation of both the maternal and embryonic components of the placental vasculature (Shweiki et al., 1993; Cullinan-Bove and Koos, 1993; Chakraborty et al., 1995; Das et al., 1997). VEGF is also required for luteal angiogenesis and associated progesterone secretion necessary to prepare the uterus for implantation (Ferrara et al., 1998). Thus, agents which inhibit the biological actions of VEGF may prove to be useful as contraceptive agents (by preventing implantation), or as an abortifacients in the early stages of gestation. The latter application might find particular use as a non-surgical intervention for the termination of ectopic pregnancies.

While the expression of VEGF receptors is largely confined to the vascular endothelium in normal reproductive tissues, Flt1 is also expressed by trophoblasts in the placenta in both humans and animals (Clark et al., 1996; He et al.,

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1999) where it has been proposed to play a role in trophoblast invasion. Interestingly, both Flt1 and KDR (Flk1) are expressed by choriocarcinoma cell line BeWo (Charnock-Jones et al., 1994), and VEGF has been shown to promote DNA synthesis and tyrosine phosphorylation of MAP kinase in these cells. Furthermore, primary and metastatic ovarian carcinomas not only to express high levels of VEGF, but-in addition to the vascular endothelium-the tumor cells themselves express KDR and/or Flt1 (Boocock et al., 1995). These 10 findings suggest that VEGF may not only be critically involved in the generation and maintenance of tumor vasculature, but that at least in some tumors of reproductive origin VEGF may subserve an autocrine role, directly supporting the survival and proliferation of the tumor cells. Thus agents which block the actions of VEGF may have particularly beneficial applications to the treatment of tumors of reproductive origin.

Methods and Results

Assessment of VEGF-Induced Uterine Hyperpermeability 20 Pregnant mare's serum gonadotrophin (PMSG) was injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats. This results in a surge of estradiol after 2 days which in turn causes an induction of VEGF in the uterus. It is reported that this induction results in hyperpermeability 25 of the uterus and an increase in uterine wet weight 6 hrs. later and, therefore, could potentially be blocked by the modified Flt receptors Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1 D2.VEGFR3D3.Fc $\Delta$ C1(a). In this in vivo model, the normal weight of the rat uterus is about 50 mg and this can be induced to 300-350 mg by PMSG. Desiccation of the tissue reveals that this is all water weight. Subcutaneous injection of Flt1(1-3)-Fc (A40),  $Flt1D2.Flk1D3.Fc\Delta C1(a)$ and Flt1D2.VEGFR3D3.FcΔC1(a) at 25 mg/kg at 1 hr after PMSG injection results in about a 50% inhibition of the increase in uterine wet weight. Increasing the dose of modified Flt receptor does not further reduce the increase in wet weight suggesting that there is a VEGF-independent component to this model. The results of this experiment are shown in FIG. 41.

Assessment of Corpus Luteum Angiogenesis Using ⁴⁰ Progesterone as a Readout

Pregnant mare's serum gonadotrophin (PMSG) is injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats. This results in a fully functioning corpus luteum containing a dense network of blood vessels after 4 days that allows for the secretion of progesterone into the blood stream in order to prepare the uterus for implantation. The induction of angiogenesis in the corpus luteum requires VEGF; therefore, blocking VEGF would result in a lack of new blood vessels and thus a lack of progesterone secreted into the blood stream. In this in vivo model, resting levels of progesterone

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are about 5 ng/ml and this can be induced to a level of 25-40 ng/ml after PMSG. Subcutaneous injection of Flt1(1-3)-Fc (A40) or Flt1D2.Flk1D3.Fc $\Delta$ C1(a) at 25 mg/kg or 5 mg/kg at 1 hr after PMSG injection results in a complete inhibition of the progesterone induction on day 4. The results of this experiment are shown in FIGS. **42**A-**42**B.

#### Example 33

#### Pharmacokinetic Analysis of Flt1(1-3)-Fc (A40) and Pegylated Flt1(1-3)-Fc

Flt1(1-3)-Fc was PEGylated with either 10 kD PEG or 20 kD PEG and tested in balb/c mice for their pharmacokinetic profile. Both PEGylated forms of Flt1(1-3)-Fc were found to have much better PK profiles than Flt1(1-3)-Fc (A40), with the  $T_{max}$  occurring at 24 hrs for the PEGylated molecules as opposed to 6 hrs for Flt1(1-3)-Fc (A40).

#### Example 34

#### VEGF165 ELISA to Test Affinity of Modified Flt1 Receptor Variants

Ten pM of VEGF165 was incubated overnight at room temperature with modified Flt1 receptor variants ranging from 160 µM to 0.1 µM. The modified Flt1 receptor variants used in this experiment were Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40), transiently expressed Flt1D2Flk1D3.FcΔC1(a), transiently expressed Flt1D2VEFGFR3D3-Fc\DeltaC1(a), Flt1-(1- $3_{NAS}$ )-FC, Flt1(1- $3_{R->C}$ )-Fc and Tie2-Fc. Flt1(1- $3_{NAS}$ )-Fc is a modified version of Flt1(1-3)-Fc in which the highly basic amino acid sequence KNKRASVRRR (SEQ ID NO:32) is replaced by NASVNGSR (SEQ ID NO:33), resulting in the incorporation of two new glycosylation sites and a net reduction of five positive charges, both with the purpose of reducing the unfavorable effects of this sequence on PK. Flt1 (1- $3_{R>C}$ )-Fc is a modification in which a single arginine (R) residue within the same basic amino acid sequence is changed to a cysteine (C) (KNKRASVRRR (SEQ D NO:36)->KNK-CASVRRR (SEQ ID NO:34)) to allow for pegylation at that residue, which could then shield the basic region from exerting its unfavorable effects on PK. After incubation the solution was transferred to a plate containing a capture antibody for VEGF165 (R&D). The amount of free VEGF165 was then determined using an antibody to report free VEGF165. This showed that the modified Flt1 receptor variant with the highest affinity for VEGF165 (determined as the lowest amount of free VEGF165) was Flt1D2Flk1D3.Fc∆C1(a), followed by Flt1(1-3)-Fc and Flt1(1-3)-Fc (A40) and then by Flt1 (1- $3_{R->C}$ )-Fc, Flt1 (1- $3_{NAS}$ )-Fc and Flt1D2VEFGFR3D3-Fc $\Delta$ C1 (a). Tie2Fc has no affinity for VEGF165.

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41

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45

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caa Gln 225	acc Thr	aat Asn	aca Thr	atc Ile	ata Ile 230	gat Asp	gtc Val	caa Gln	ata Ile	agc Ser 235	aca Thr	cca Pro	cgc Arg	cca Pro	gtc Val 240	720		
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ccc Pro	ttg Leu	aac Asn	acg Thr 260	aga Arg	gtt Val	caa Gln	atg Met	acc Thr 265	tgg Trp	agt Ser	tac Tyr	cct Pro	gat Asp 270	gaa Glu	att Ile	816		
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tcc	ttc	ttc	ctc	tac	agc	aag	ctc	acc	gtg	gac	aag	agc	agg	tgg	cag	1584		

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cag Gln	999 Gly 530	aac Asn	gtc Val	ttc Phe	tca Ser	tgc Cys 535	tcc Ser	gtg Val	atg Met	cat His	gag Glu 540	gct Ala	ctg Leu	cac His	aac Asn	1632	
cac His 545	tac Tyr	acg Thr	cag Gln	aag Lys	agc Ser 550	ctc Leu	tcc Ser	ctg Leu	tct Ser	ccg Pro 555	ggt Gly	aaa Lys	tga			1674	
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Glu	Leu	Ser 35	Leu	Lys	Gly	Thr	Gln 40	His	Ile	Met	Gln	Ala 45	Gly	Gln	Thr		
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Сүз	Gly	Arg	Asn	Gly 85	ГЛЗ	Gln	Phe	Суз	Ser 90	Thr	Leu	Thr	Leu	Asn 95	Thr		
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Thr	Ser	Pro	Asn	Ile 165	Thr	Val	Thr	Leu	Lys 170	Lys	Phe	Pro	Leu	Asp 175	Thr		
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Ala	Thr 210	Val	Asn	Gly	His	Leu 215	Tyr	Lys	Thr	Asn	Tyr 220	Leu	Thr	His	Arg		
Gln 225	Thr	Asn	Thr	Ile	Ile 230	Asp	Val	Gln	Ile	Ser 235	Thr	Pro	Arg	Pro	Val 240		
ГÀа	Leu	Leu	Arg	Gly 245	His	Thr	Leu	Val	Leu 250	Asn	Cys	Thr	Ala	Thr 255	Thr		
Pro	Leu	Asn	Thr 260	Arg	Val	Gln	Met	Thr 265	Trp	Ser	Tyr	Pro	Asp 270	Glu	Ile		
Asp	Gln	Ser 275	Asn	Ser	His	Ala	Asn 280	Ile	Phe	Tyr	Ser	Val 285	Leu	Thr	Ile		
Asp	Lys 290	Met	Gln	Asn	Lys	Asp 295	Lys	Gly	Leu	Tyr	Thr 300	Сүз	Arg	Val	Arg		
Ser 305	Gly	Pro	Ser	Phe	Lys 310	Ser	Val	Asn	Thr	Ser 315	Val	His	Ile	Tyr	Asp 320		
Lys	Ala	Gly	Pro	Gly	Glu	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr	Cys		

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				325					330					335			
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Pro	Arg	Glu	Glu	Gln 405	Tyr	Asn	Ser	Thr	Tyr 410	Arg	Val	Val	Ser	Val 415	Leu		
Thr	Val	Leu	His 420	Gln	Asp	Trp	Leu	Asn 425	Gly	Lys	Glu	Tyr	Lys 430	Суз	Lys		
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Pro	Glu	Asn	Asn	405 Tyr	Lys	Thr	Thr	Pro	Pro	Val	Leu	Asp	Ser	Asp	Gly		
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Met 1	Val	Ser	Tyr	- 59 Trp 5	Asp	Thr	Gly	Val	Leu 10	Leu	CAa	Ala	Leu	Leu 15	Ser		
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atg Mct	tac	agt	gaa	atc	CCC	gaa	att	ata	cac	atg Mot	act	gaa	gga	agg	gag	144	
Met	ıyr	ser 35	GIU	11e	Pro	GIU	11e 40	цте	ніз	Met	Thr	45	σту	Arg	GIU		
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65					70		-+	a+-	+	75			+		80	000	
ugg Trp	yac Asp	ayt Ser	aya Arg	aag Lys 85	yyc Gly	Phe	atC Ile	ata Ile	Ser 90	aat Asn	yca Ala	acg Thr	tac Tyr	aaa Lys 95	yaa Glu	288	
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ata Ile	agc Ser 130	aca Thr	cca Pro	cgc Arg	cca Pro	gtc Val 135	aaa Lys	tta Leu	ctt Leu	aga Arg	ggc Gly 140	cat His	act Thr	ctt Leu	gtc Val	432
ctc Leu 145	aat Asn	tgt Cys	act Thr	gct Ala	acc Thr 150	act Thr	ccc Pro	ttg Leu	aac Asn	acg Thr 155	aga Arg	gtt Val	caa Gln	atg Met	acc Thr 160	480
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Tyr 305	Arg	Val	Val	Ser	Val 310	Leu	Thr	Val	Leu	His 315	Gln	Asp	Trp	Leu	Asn 320	
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											-	con	tin	ued		
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atg Met	gtc Val	agc Ser	tac Tvr	tgg Trp	gac Asp	acc Thr	ggg	gtc Val	ctg	ctg Leu	tgc Cva	gcg Ala	ctg Leu	ctc	agc Ser	48
1		~~1	-1-	5		1	3± Y	. 41	10	Luu	-19		Lu	15	~~1	
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atg Met	tac Tyr	agt Ser 35	gaa Glu	atc Ile	ccc Pro	gaa Glu	att Ile 40	ata Ile	cac His	atg Met	act Thr	gaa Glu 45	gga Gly	agg Arg	gag Glu	144
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ata Ile	elà aaa	ctt Leu	ctg Leu 100	acc Thr	tgt Cys	gaa Glu	gca Ala	aca Thr 105	gtc Val	aat Asn	д1у 999	cat His	ttg Leu 110	tat Tyr	aag Lys	336
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agg Arg	agt Ser 210	gga Gly	cca Pro	tca Ser	ttc Phe	aaa Lys 215	tct Ser	gtt Val	aac Asn	acc Thr	tca Ser 220	gtg Val	cat His	ata Ile	tat Tyr	672
gat Asp 225	aaa Lys	gca Ala	ggc Gly	ccg Pro	ggc Gly 230	gag Glu	ccc Pro	aaa Lys	tct Ser	tgt Cys 235	gac Asp	ааа Lys	act Thr	cac His	aca Thr 240	720
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Met	Tyr	Ser 35	Glu	Ile	Pro	Glu	Ile 40	Ile	His	Met	Thr	Glu 45	Gly	Arg	Glu
Leu	Val 50	Ile	Pro	Суз	Arg	Val 55	Thr	Ser	Pro	Asn	Ile 60	Thr	Val	Thr	Leu
Lys 65	Lys	Phe	Pro	Leu	Asp 70	Thr	Leu	Ile	Pro	Asp 75	Gly	Lys	Arg	Ile	Ile 80
Trp	Asp	Ser	Arg	Lys 85	Gly	Phe	Ile	Ile	Ser 90	Asn	Ala	Thr	Tyr	Lys 95	Glu
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Trp	Ser	Tyr	Pro	Asp 165	Glu	rÅa	Asn	ГЛа	Arg 170	Ala	Ser	Val	Arg	Arg 175	Arg
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Ile	Asp	Lys 195	Met	Gln	Asn	Lys	Asp 200	Lys	Gly	Leu	Tyr	Thr 205	Суз	Arg	Val
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Сүз	Pro	Pro	Сув	Pro 245	Ala	Pro	Glu	Leu	Leu 250	Gly	Gly	Pro	Ser	Val 255	Phe
Leu	Phe	Pro	Pro 260	Lys	Pro	Lys	Asp	Thr 265	Leu	Met	Ile	Ser	Arg 270	Thr	Pro
Glu	Val	Thr 275	Сув	Val	Val	Val	Asp 280	Val	Ser	His	Glu	Asp 285	Pro	Glu	Val
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Lys	Val	Ser	Asn 340	Lys	Ala	Leu	Pro	Ala 345	Pro	Ile	Glu	Lys	Thr 350	Ile	Ser
Lys	Ala	Lys 355	Gly	Gln	Pro	Arg	Glu 360	Pro	Gln	Val	Tyr	Thr 365	Leu	Pro	Pro
Ser	Arg 370	Asp	Glu	Leu	Thr	Lys 375	Asn	Gln	Val	Ser	Leu 380	Thr	Cys	Leu	Val
Lys 385	Gly	Phe	Tyr	Pro	Ser 390	Asp	Ile	Ala	Val	Glu 395	Trp	Glu	Ser	Asn	Gly 400
Gln	Pro	Glu	Asn	Asn 405	Tyr	rÀa	Thr	Thr	Pro 410	Pro	Val	Leu	Asp	Ser 415	Asp

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Gln	Gln	Gly 435	Asn	Val	Phe	Ser	Cys 440	Ser	Val	Met	His	Glu 445	Ala	Leu	His		
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tgt Cys	gga Gly	aga Arg	aat Asn	ggc Gly 85	aaa Lys	caa Gln	ttc Phe	tgc Cys	agt Ser 90	act Thr	tta Leu	acc Thr	ttg Leu	aac Asn 95	aca Thr	288	
gct Ala	caa Gln	gca Ala	aac Asn 100	cac His	act Thr	ggc Gly	ttc Phe	tac Tyr 105	agc Ser	tgc Cys	aaa Lys	tat Tyr	cta Leu 110	gct Ala	gta Val	336	
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 160

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Cys Se 545	er Val	Met	His	Glu 550	Ala	Leu	His	Asn	His 555	Tyr	Thr	Gln	Lys	Ser 560				
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Val Val Leu Ser

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We claim:

1. A dimeric vascular endothelial growth factor (VEGF) antagonist, comprising two fusion proteins, each fusion protein consisting of (a) a VEGF receptor component having immunoglobulin-like (Ig) domains consisting of an Ig domain 2 of a first VEGF receptor human Flt1 and Ig domain 3 of a second VEGF receptor human Flk1; and (b) a multimerizing component.

**2**. The dimeric VEGF antagonist of claim **1**, wherein the ²⁰ multimerizing component comprises an immunoglobulin domain.

**3**. The dimeric VEGF antagonist of claim **2**, wherein the immunoglobulin domain is chosen from the Fc domain of IgG and the heavy chain of IgG.

**4**. The dimeric VEGF antagonist of claim **3**, wherein each fusion protein comprises amino acids 27-458 of the amino acid sequence shown in SEQ ID NO:16.

**5**. A dimeric vascular endothelial growth factor (VEGF) antagonist, comprising two fusion proteins, each fusion protein consisting of (a) a VEGF receptor component having

immunoglobulin-like (Ig) domains consisting of an Ig domain 2 of a first VEGF receptor human Flt1 and Ig domain 3 of a second VEGF receptor human Flk1; and (b) an Fc domain of IgG.

6. A composition comprising a VEGF antagonist and a carrier, wherein the VEGF antagonist comprises two fusion proteins, each fusion protein consisting of (a) a VEGF receptor component having immunoglobulin-like (Ig) domains consisting of an Ig domain 2 of a first VEGF receptor human Flt1 and Ig domain 3 of a second VEGF receptor human Flt1; and (b) a multimerizing component.

7. The composition of claim 6, wherein the multimerizing component comprises an immunoglobulin domain.

**8**. The composition of claim **7**, wherein the immunoglobulin domain is an Fc domain of IgG.

**9**. The composition of claim **8**, wherein each fusion protein comprises amino acids 27-458 of the amino acid sequence of SEQ ID NO:16.

* * * * *