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### (12) United States Patent

### Papadopoulos et al.

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# (54) CELL CULTURE COMPOSITIONS CAPABLE OF PRODUCING A VEGF-BINDING FUSION POLYPEPTIDE

(75) Inventors: Nicholas J. Papadopoulos,

Lagrangeville, NY (US); Samuel Davis, New York, NY (US); George D. Yancopoulos, Yorktown Heights, NY

(US)

(73) Assignee: Regeneron Pharmaceuticals, Inc.,

Tarrytown, NY (US)

(\*) Notice: Subject to any disclaimer, the term of this

patent is extended or adjusted under 35

U.S.C. 154(b) by 0 days.

This patent is subject to a terminal dis-

claimer.

(21) Appl. No.: 13/106,910

(22) Filed: May 13, 2011

#### (65) **Prior Publication Data**

US 2012/0064621 A1 Mar. 15, 2012

#### Related U.S. Application Data

- (60) Continuation of application No. 12/334,927, filed on Dec. 15, 2008, now Pat. No. 7,964,377, which is a continuation of application No. 12/102,648, filed on Apr. 14, 2008, now Pat. No. 7,524,499, which is a division 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.
- (60) Provisional application No. 60/138,133, filed on Jun. 8, 1999.

(51)	Int. Cl.	
	C12N 5/10	(2006.01)
	C12N 5/16	(2006.01)
	C12N 15/62	(2006.01)
	C12N 15/63	(2006.01)
	C07H 21/04	(2006.01)

- (58) **Field of Classification Search** ....... None See application file for complete search history.

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#### (57) ABSTRACT

The present invention provides cell culture compositions capable of producing fusion polypeptides that bind vascular endothelial growth factor (VEGF). The cell culture compositions of the invention comprise cells which contain an expression vector comprising a nucleic acid molecule encoding a fusion polypeptide that binds VEGF. The fusion polypeptides may comprise a VEGF receptor component having an immunoglobulin-like (Ig) domain 2 of a first VEGF receptor, an Ig domain 3 of a second VEGF receptor, and a multimerizing component.

#### 12 Claims, 55 Drawing Sheets

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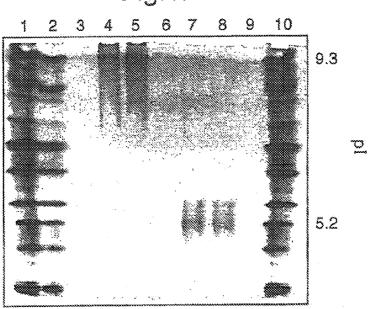
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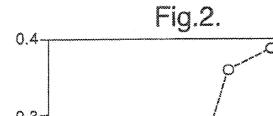
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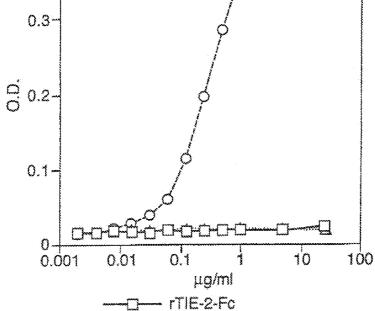
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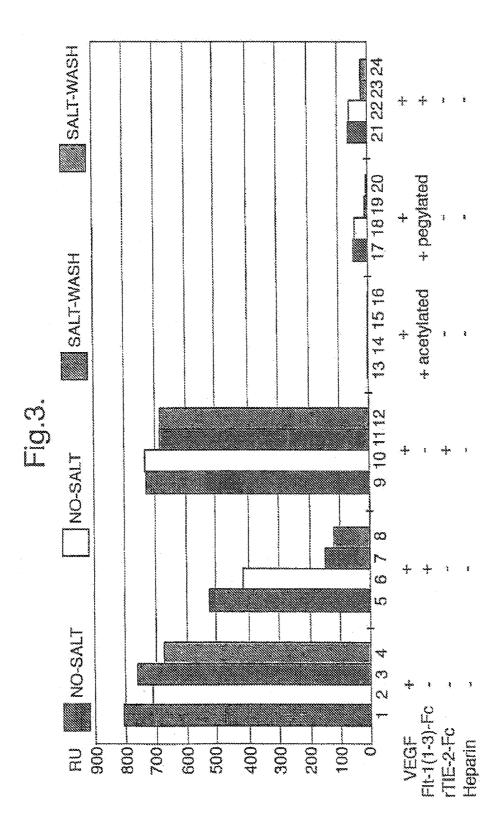
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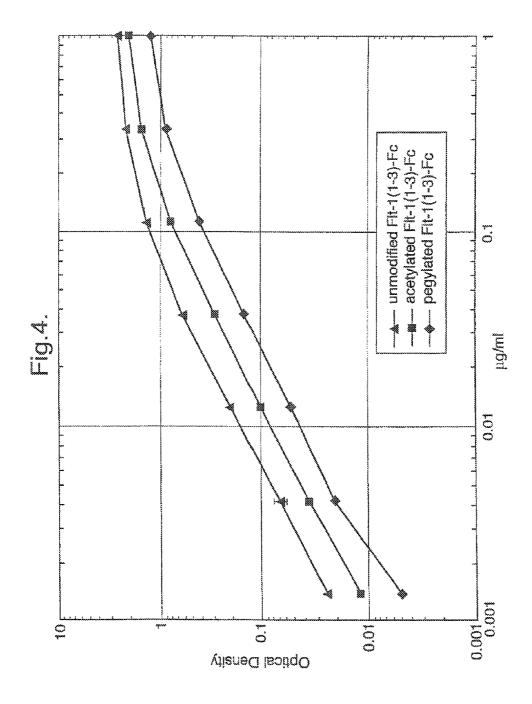












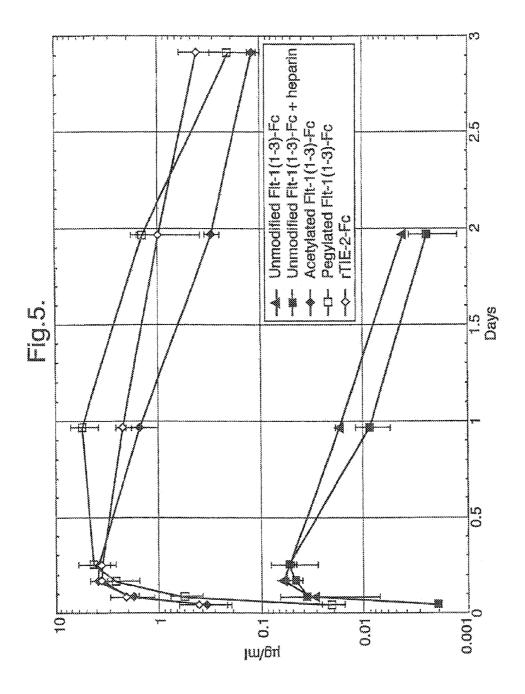


Fig.6A.

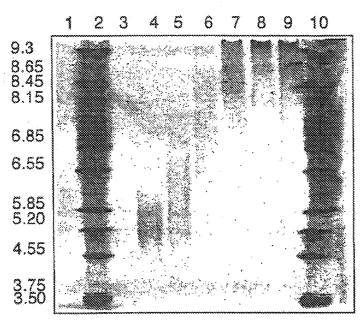
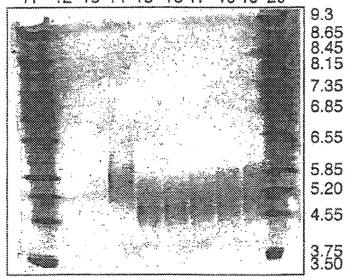
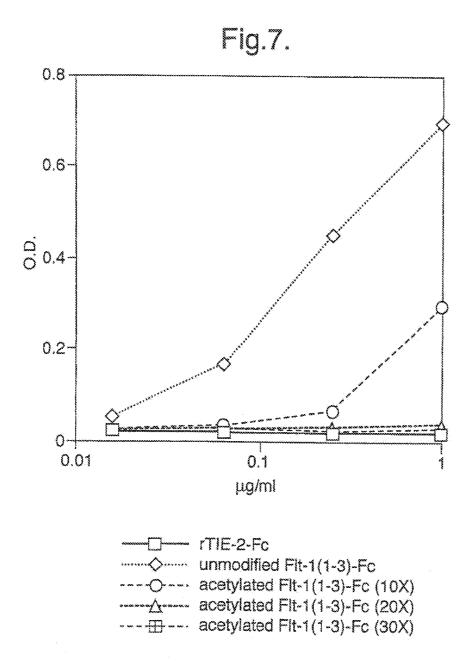
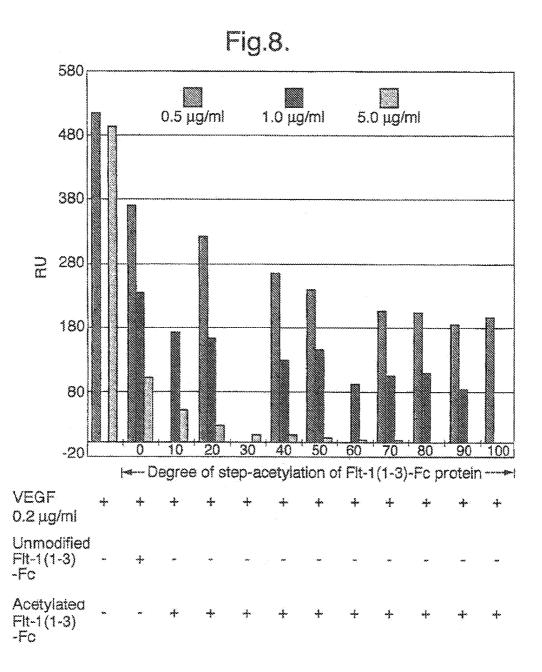


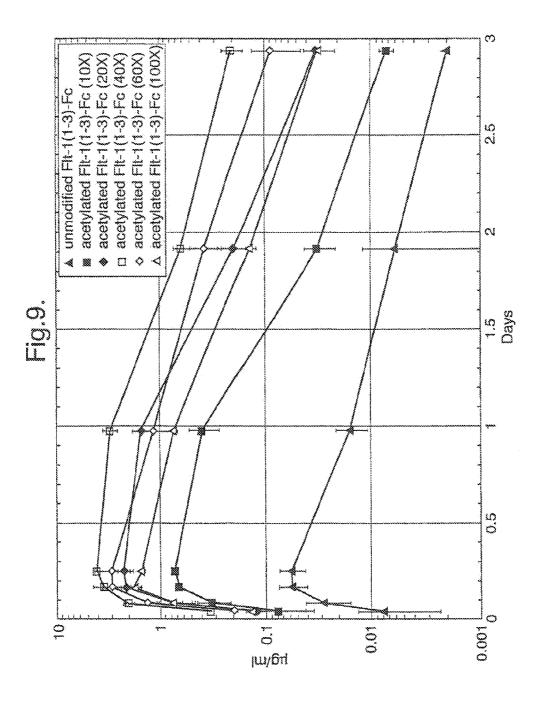
Fig.6B.

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### Fig. 10A.

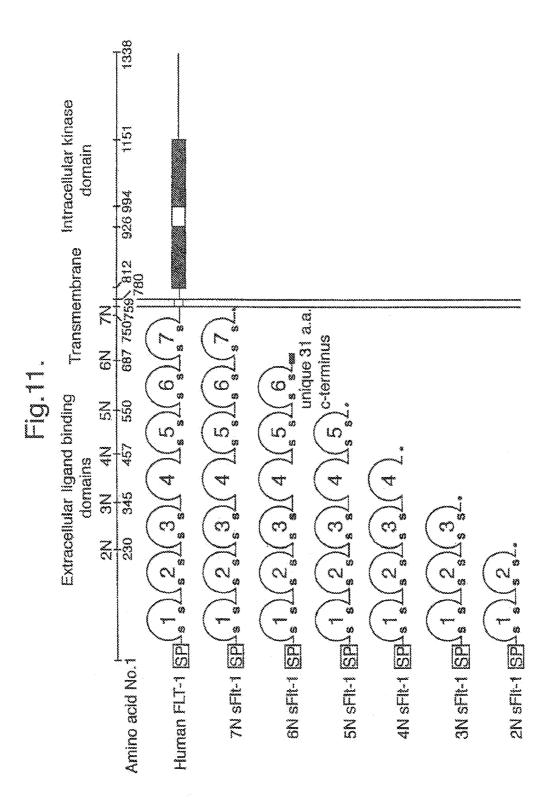
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### Fig. 10D.



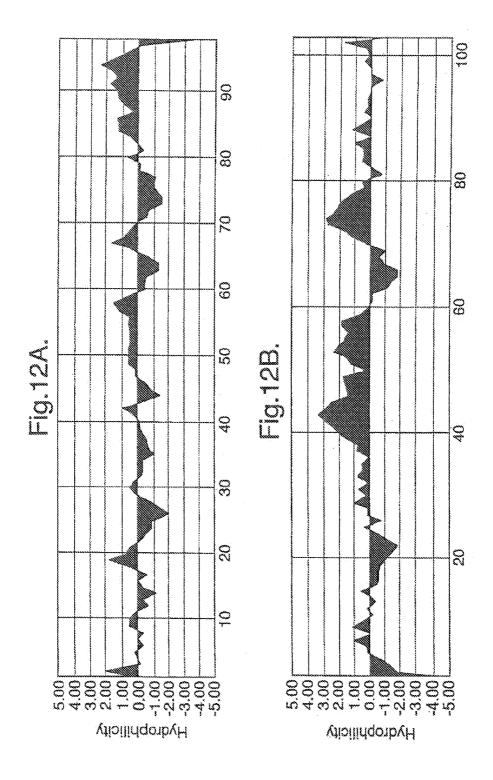


Fig. 13A.

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### Fig. 13C. 1110 1120 1130 1140 CTC ATG ATC TOC 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 Fro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp> 1180 1190 1170 CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC 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 Cly Val Glu Val His Asn Ala Lys Thr Lys> 1220 1230 1240 1250 1260 \* \* \* \* \* \* \* \* \* CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC GGT 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 GTC Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His> 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> 1340 1350 1360 1370 1340 CCC ATC GAG ANA NCC ATC TCC ANA GCC ANA GGG CAG CCC CGA GNA CCA CAG GTG TRC ACC COG TAG CTC TIT TOG TAG AGG TIT COG TIT CCC GTC GOG GCT CIT GGT GTC CAC ATG TGG Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thu> 1390 1400 1410 1420 OTG CCC CCA TCC CCC 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 Fro Pro Ser Arg Asp Glu Leu Tur Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys> 1460 1470 1480 \* \* \* \* \* \* \* 1490 1460 OGC TIC TAT COO AGO GAO ATO GOO GING GAG IGG GAG AGO AAT GGG CAG COO GAG AAC AAC COS AAS ATA GGG TOG CTG TAG CGG CAC CTC ACC CTC TOG TTA CCC GTC GGC CTC TNG TTG Gly Fhe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn> 1520 1530 1540 1550 \* \* \* \* \* \* \* TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ATC TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG GAG ATC TCG TTC GAG Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Fhe Phe Leu Tyr Ser Lys Leu> 1570 1580 1590 1600 \* \* \* \* \* \* \* \* \* ACC GIG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG

TOG CAC CTG TTC TCC 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> U.S. Patent Jan. 1, 2013 Sheet 18 of 55 US 8,343,737 B2

## Fig. 13D.

#### Fig. 14A. ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GGG 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 Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu> 100 90 33.0 80 ACA GGA TOT ACT TOO GGA GGT AGA COT TTO GTA GAG ATG TAC AGT GAA ATC COO GAA ATT TOT CCT AGA TOA AGG COT COA TOT GGA AAG CAT CIC TAC AIG TOA CIT TAG GGG CIT TAA Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Vel Glu Met Tyr Ser Glu Ile Pro Glu Ile> 160 150 140 ATA CAC ATC ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT ACG TCA CCT AAC ATC THAT GTG THE TGA CTT CUT TOO CTC GRG 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> 240 200 . 220 230 210 ACT GIT ACT TIA AAA AAG TIT CCA CIT GAC ACT TIG ATC CCT GAT GGA AAA CCC ATA ATC TOA CAA TOA AAT TIT TIC AAA GOT GAA CIG TOA AAC TAG GOA CIA CCT TIT GCG TAT TAG Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile> 300 270 280 290 250 TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA GAA ATA GGG CTT CTG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT CTT TAT CCC GAA GAC Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu> 360 310 320 ACC TOT GAA OCA ACA OTC AAT GGG CAT TTG TAT AAG ACA AAC TAT CTC ACA CAT GGA CAA TOG ACA CTT COT TOT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA GAG TGT GTA GCT GTT Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Glm> 420 400 410 380 376 4 ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC AAA TTA CTT AGA GGC TOG TTA TOT TAG TAT CTA CAG GIT TAT TOG TOT GOT GOG GOT CAG TIT AAT GAA TOT COG Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly> 460 470 450 440 \* CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG AGA GTT CAA ATG ACC GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG TGA GGG AAC TTG TGC TCT CAA GTT TAC TGG His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr Arg Val Gln Met Thr> 540 510 520 530 TOG AGT TAC COT GAT GAA ATT GAD DAA AGC AAT TOD DAT GOD AAC ATA TTO TAC AGT GTT ACC TOA ATG GEA CTA CTT TAA CTG GTT TOG TTA AGG GTA CGG TTG TAT AAG ATG TOA CAA Trp Ser Tyr Pro Asp Glu Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val>

### Fig. 14B.

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The Pro Glu Val The Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Photometry  850 860 870 880 890 900  AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG GGG GAG GAG CAG  TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC  Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Als Lys The Lys Pro Arg Glu Glu Glu Cln  910 920 930 940 950 960  TAC AAC ACC ACG TAC CCT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT  ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GTC TTA  Tyr Asn Ser The Tyr Arg Val Val Ser Val Leu The Val Leu His Gln Asp Trp Leu Asn>  970 980 990 1000 1010 1020  * * * * * * * * * * * * * * * * * * *	ACC	CCI	GAG	GTC	ACA	. TGC	GTG	GTG	GTG	GAC	GTG	AGC	CAC	GAA	GAC	CCT	CAL	er.	energy mmm	7.1%
850 860 870 880 890 900  AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC AER TTD TYT VAL AED GLY VAL GLU VAL HIS AER ALS LYE TET LYE PTO ATG GLU GLU GLU>  910 920 930 940 950 960  TAC AAC ACC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG ATG TTG TCG TCC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TAC TYT AER SET THT TYT ATG VAL VAL SET VAL LEU THT VAL LEU HIS GLU AED TTP LEU AER>  970 980 990 1000 1010 1020  GCC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCG GGC TAG CTC TTT TGG CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG GLY LYE GLU TYT LYE CYE LYE VAL SET AER LYE ALS LEU PTO ALS PTO LIE GLU LYE THT>  1030 1040 1050 1060 1070 1080  ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG TAG GAG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GGG GGT AGC CCC TTG CCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG TAG GAG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GGG GGT AGC CCC TTG CCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG TAG GAG TTT CGG TTT CCC GTC GGG GCT CTT GGT CTC CAC ATG TGG GGG GGT AGC CCC TAG GAG TTT CGG TTT CCC GTC GGG GCT CTT GGT CTC CAC ATG TGG GGG GGT AGC CCC TAG GAG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GGG GGT AGC CCC TAG GAG TTT CGG TTT CCC GTC GGG GGT TTT CTT GGT GTC CAC ATG TGG GGG GGT AGC CCC	TGG.	GGA	CIC	CAG	TE	ACG	CAC	CAC	CAC	CIG	CAC	TUG	616	Crr	CHG	CKAR	L'XU	الانسا	Yara	Mines.
AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ASN TTP TYY Val ASP Gly Val Glu Val His Asn Als Lys Thr Lys Pro Arg Glu Glu Glu>  910 920 930 940 950 960  TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA TYT ASN Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp TTP Leu Asn>  970 980 990 1000 1010 1020  GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr>  ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGC GAG GGT AGG GCC TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG GAG GGG GGT AGG GCC TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG GAG GGG GGT AGG GCC	Thr	520	Glu	Val	Thr	Cys	Val	. Val	Val	Asp	Vai	. Ser	HLS	GTA	ASD	KID	GIU	AST	ry	Files
AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ASN TTP TYY Val ASP Gly Val Glu Val His Asn Als Lys Thr Lys Pro Arg Glu Glu Glu>  910 920 930 940 950 960  TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA TYT ASN Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp TTP Leu Asn>  970 980 990 1000 1010 1020  GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr>  ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGC GAG GGT AGG GCC TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG GAG GGG GGT AGG GCC TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG GAG GGG GGT AGG GCC			2				مامم			2000				o n			იიი			one
AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GGC AAG ACA AAG CCG CGG GAG GAG CAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ASN TTD TYY Val ASD Gly Val Glu Val His ASN Als Lys Thir Lys Pro Arg Glu Glu Glu>  910 920 930 940 950 960  TAC AAC AGG ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT ATG TTG TGG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA TYT ASN Ser Thir Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp TTP Leu Asn>  970 980 990 1000 1010 1020  GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Als Leu Pro Als Pro Ile Glu Lys Thir>  1030 1040 1050 1050 1060 1070 1080  ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG TAG AGG TTT CGC TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG GAG GGG GGT AGG GCC TAG AGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG GAG GGG GGT AGG GCC TAG AGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG GAG GGG GGT AGG GCC TAG AGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGC GAG GGT AGG GCC			8						۵.			· du	\$						*	
TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC  Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Glu>  910 920 930 940 950 960  *  TAC AAC ACC ACC TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT  ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GTG TAC  Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn>  970 980 990 1000 1010 1020  *  GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC  CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG  Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr>  1030 1040 1050 1060 1070 1080  *  ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG  TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC  TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC								. ~					3 5 73			ANC		CD ACT		
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910 920 930 940 950 960  * * * * * * * * * * * * * * * * * * *	J. T.	ALC	All	CAC	CAG	الله الله الله الله الله الله الله الله	ومغيا	wrw.	tion 1	era w	9 man	1 2 1 m	Tan	Triba.	Tien	Parties Parties	S was	(234)	Clu	เรากร
TAC AND AGE AGE TAC CGT GTG GTC AGE GTC CTC AGE GTC CTG CAC CAG GAC TGG CTG AAT ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA  Tyt Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn>  970  980  950  1000  1010  1020  66C AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr>  1030  1040  1050  1060  1070  1080  ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG TAG AGG TTT CGC GTC GCG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC TAG AGG TTT CGC TTT CCC GTC GCG GCT CTC CCC GAC GAC GCC	ASD	.rr.b	лУх	AST	Asp	e erry	. Agr	. Later	val	nie	ASST.	i shire	ກັສ	4014	. My to	220	er A	www	Sec	Acousto.
TAC AND AGE AGE TAC CGT GTG GTC AGE GTC CTC AGE GTC CTG CAC CAG GAC TGG CTG AAT ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA  Tyt Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn>  970  980  950  1000  1010  1020  66C AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr>  1030  1040  1050  1060  1070  1080  ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG TAG AGG TTT CGC GTC GCG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC TAG AGG TTT CGC TTT CCC GTC GCG GCT CTC CCC GAC GAC GCC			ň	š A			ann.			ত্রগ			q	40			950			960
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ATC TIC TCC TCC ATC GCA CAC CAC TCC CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA  TYT ASN SET THE TYT ATG VAI VAI SET VAI LEU THE VAI LEU HIS GIN ASP TTP LEU ASN>  970 980 990 1000 1010 1020  * * * * * * * * * * * * * * * * * * *	mace	220	אלי) א		mac	C COCOLO		מיושה:		Called	(3)(	204.	: 090	CTC	COACO	CAG	GAC	TGG	CIG	AAT
Tyr Asn Ser Thr Tyr Arg Vel Vel Ser Vel Leu Thr Vel Leu His Gin Asp Trp Leu Asn>  970  980  990  1000  1010  1020  *  GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG Gly Lys Glu Tyr Lys Cys Lys Vel Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr>  1030  1040  1050  1050  1060  1070  1080  *  ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG TAG AGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC																				
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CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr>  1030 1040 1050 1050 1070 1080  * * * * * * * * * * * * * * * * * * *		*		*	4	r	*		ŵ,			*		sé.	nk		*		*	*
CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr>  1030 1040 1050 1050 1070 1080  * * * * * * * * * * * * * * * * * * *	cace:	מממ	്വുമ	ጣልጦ	מממ י	nese e	. AAC	e Gre	ncc	CAA.	AA	A GCC	cro	CO	GCC	ccc	ATC	GAG	AAF	ACC
Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr>  1030 1040 1050 1060 1070 1080  * * * * * * * * * * * * * * * * * * *	CCC	JAM	CW	YPA	dxlk	ACC	TTO	CAG	AGG	TIC	T	rccc	GAG	GGI	CGG	GGG	TAG	cro	TH	TGG
ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG TAG AGG TTT CGC TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC	Gly	Lvs	Glu	TVI	Lvs	Cys	Lys	val	Ser	Asr	Lys	a Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lye	Thr:>
ATC TOO AAA GOO AAA GOO CAG COO CGA GAA COA CAG GTG TAC ACC CTG COO CCA TOO CGO TAG AGG TIT CGG TIT COO GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC				- 4		•														
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The Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg>	TAG	AGG	TT	CGC	TT	r add	Gr(	GGG	GC7	con	r GG	r GT	CAC	TEA :	TGC	GAC	GGC	GG	' AGC	a GCC
	Ile	Ser	Lys	Ala	Lye	s Gly	/ Gls	r Pro	) Arg	gCli	ı Pro	o Glr	ı Val	Tyr	: Thu	Leu	1 Pro	) Pro	Ser	: Arg>

### Fig.14C.

1110 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 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 3.230 1240 1250 1260 CCC GIG CTG GAC TCC GAC GGC TCC TIC 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 1290 1300 1310 1320 1280 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 1340 1350 TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA ATG TOO GTO TTO TOG GAG AGG GAC AGA GGO COA TTT ACT Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys \*\*\*>

### Fig. 15A.

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							CIC												
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## Fig. 15B.

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### Fig. 15C.

1120 1100 1110 1090 CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu> 1160 1170 1180 1190 ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG TOG ACC GAC CAG TTT CCC AAG ATA GGG TCC CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly> 1220 1230 1240 1250 1,210 CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC GGC CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG Gin Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe> 1280 1290 1300 1310 1320 1270 CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC CAG ATC TCC TTC CAG TCG CAC CTG TTC TCG TCC ACC GTC GTC CCC TTC CAG AAG AGT ACC Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Fhe Ser Cys> 1340 1350 1360 1330 TOO GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG GAG AGG GAC AGA GGC Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro> GGT AAA TGA CCA TIT ACT Gly Lys \*\*\*>

### Fig. 16A.

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ATG GT																			
TAC C																			
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TGT G	KIA	AGA	AAT	GGC	AAA	CAA	TTC	TGC	AGT	ACT	TTA	ACC	TTG	AAC	ACA	GCT	CAA	GCA	AAC
ACA C																			
Cys G	31.y	Arg	Asn	Gly	Lys	Gln	Phe	Cys	Ser	Thu	Leu	Thr	Leu	Asn	Thr	Ala	Gln	Ala	Asn>
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CAC A																			
GIG I																			
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### Fig. 16B.

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CIT	TAT	CCC	GAA	GAC	ngg	ACA	CTT	CGT	TGT	CAG	TTA	$\mathbb{C}\mathbb{C}\mathbb{C}$	GTA	AAC	ATA	TIC	TGT	LIG	ATA
Glu	Ile	Gly	Leu	ren	Thr	Cys	Glu	Ala	Thr	Val	Asn	Gly	His	Leu	Tyr	Lys	Thr	han	LALS
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Leu	Thr	His	Arq	Gln	Thr	Asn	Thr	Ile	Ile	Asp	Val	Gln	Ile	Sex	Thr	Pro	Arg	Pro	Val>
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AGA	GII	CAP	ATC	ACC	TGG	agt	TAC	CCI	GAT	GAA	AAA	. AAI	r aac	AAC	GCI	acc.	GTA	AGG	CGA
TCT	CAA	GI	TAC	103	ACC	TCA	<b>ETTA</b>	GGA	CIN	CTI	TIT	LI	IN	TTC	CGA	AGG	CAS	, acc	cor
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GCT	TAP	cro	GTY	TO	ATT :	AGG	GTZ	000	TT	TAT	DAA 1	ma i	a acu	A CAP	(GA)	l TGI	TAI	A CIC	TTT
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CIO	TT	r ra	A GT	G IG	TAC	3 GG	r GG	CAC	G GG	ar Ca	er GG	a Ci	T GA	G GA	C CC	c cc	at Co	K AC	T CAG
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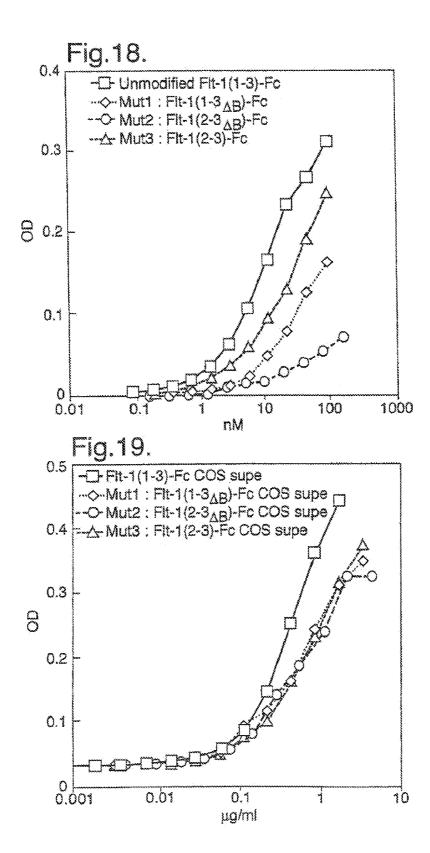
## Fig. 16C.

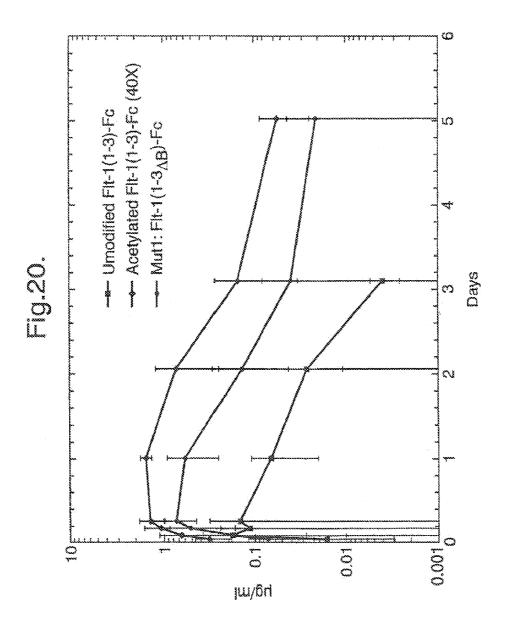
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### Fig. 16D.

Leu Ser Leu Ser Pro Gly Lys \*\*\*>

Fig.17. 1.0 μg/ml 0.25 μg/ml 0.5 μg/ml 250 200 150 2 100 50 VEGF (0.1 μg/ml) COS supe Mut4 : FIt-1(1-3<sub>H→N</sub>)-Fc + 4 COS supe Mutf::Fit-1(1-3<sub>AB</sub>)-Fc N-1(1-3)-Fc Purified unmodified Fit-1(1-3)-Fc Purified acetylated Fit-1(1-3)-Fc COS supe unmodified FIt-1(1-3)-Fc





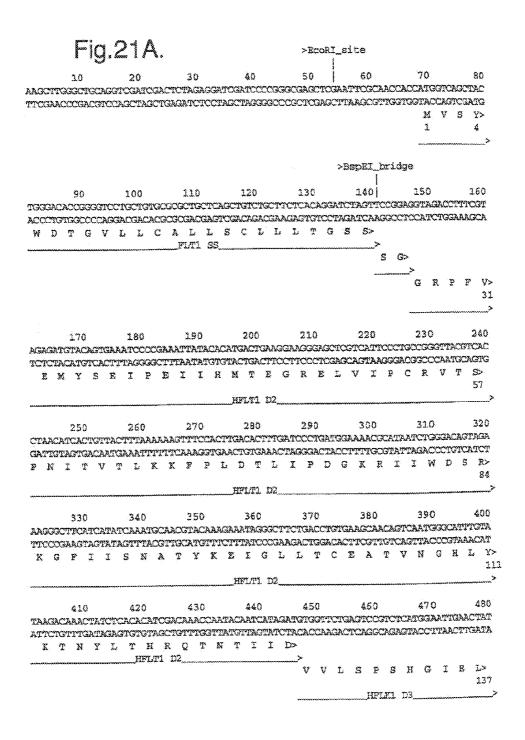


Fig.21B. 500 510 520 530 540 CTOTTGEAGAAAGCTTGTCTTAAATTGTACAGCAAGAACTGATCTAAATTGTGGGATTGACTTCAACTGGGATACCCT GACAACCTCTTTTCGAACAGAATTTAACATGTCCTTGACTTGATTTACACCCCTAACTTGACCTTATGGGA SVGERLVLNCTARTELNVGIDFNWBYP> HFLK1 D3 620 630 570 590 600 610 TOTTOGRAGORITORGORITARGARACTIGIARACCGAGACCTRARARACCCAGTOTGGGAGTGAGAGAGAGAATTTTIGAG AGAAGCTTCGTAGTCGTATTCTTTGAACATTTGGCTCTGGATTTTTGGGTCAGACCCTCACTCTACTTCTTAAAAACTC SSRHQHKKLVNRDLKTQSGSEMKKPLS> 191 HFLK1 D3\_\_\_ 700 670 680 690 660 CACCTTACTATAGATGGTGTAACCCGGAGTGACCAAGGATTGTACACCTGTGCAGCATCCAGTGGGCTGATGACCAAGA GTGGAATTGATATCTACCACATTGGGCCTCACTGGTTCCTAACATGTGGACACGTCGTAGGTCACCGGACTACTGGTTCT TLTIDGVTRSDQGLYTCAASSGLMTK> 227 HFLK1 D3 >Srf\_Bridge\_ 750 760 AGAACAGCACATTTGTCAGGGTCCATGAAAAGGGGCCCGGGGGACAAAACTCACACATGCCCCACCGTGCCCAGCACCACCTGAA TCTTTTCGTGTAAACAGTCCCAGGTACTTTTCCCGGGCCCGCTGTTTTGAGTGTGTACGGGTGGCACGGGTCGTGGACTT K NSTFVRVHEK> HFLK1 D3 G P G> DKTHTCPPCPAPE> 244 \_PCACI(A)\_ 87.0 830 840 850 860 CTCCTROSGRBACCCTCAGTCTTCCTCTTCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCAC GAGGACCCCCTGGCAGTCAGAAGGAGAAGGGGGGTTTTGGGTTCCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAGTG LLGGPSVFLFPPKPKDTLMISRTFEVT> 271 FCACL(A) 920 930 940 93.0 900 890 TACGCACCACCACCACCACCACTCGCTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCACCTCCACGTATTAC C V V V D V S H E D P E V K F N W Y V D G V E V H IS \_FCΔC1 (A) \_\_\_ 990 1000 1010 1020 980 CCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGTCTACCGTCCTCACCGTCCTGCACCAGGACTGG GGTTCTGTTTCGGCGCCCTCCTCGTCATGTTGTCGTGCATGGCACACCACTCGCAGGAGTGGCAGGACGTGGTCCTGACC AKTEFREEQYNSTYRVVSVLTVLHQDW FCAC1 (A)

Fig.21C.

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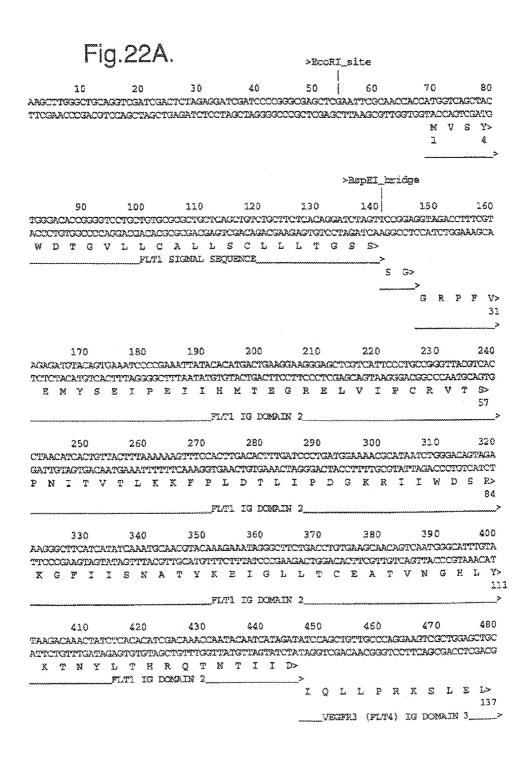
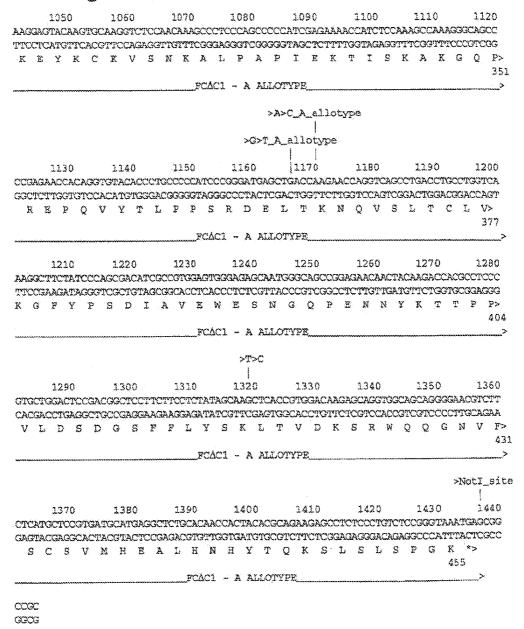
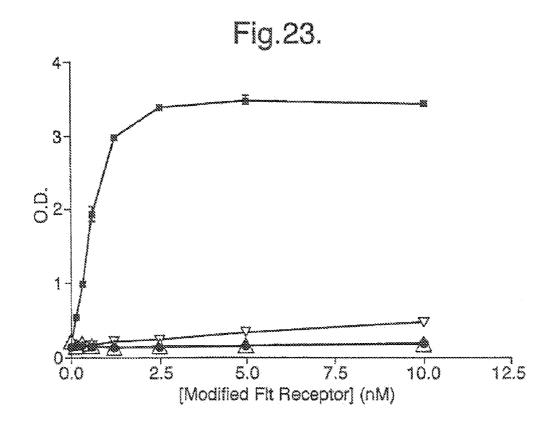


Fig.22B.

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Fig.22C.





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- △Flt1D2VEGFR3D3 FcdeltaC1(a)
- ♥ TIE2-Fc
- Flt1(1-3)-Fc

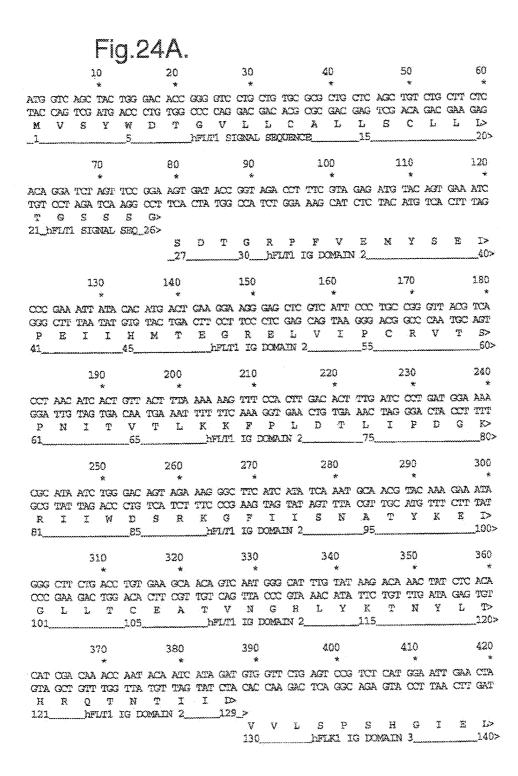


Fig.24B.

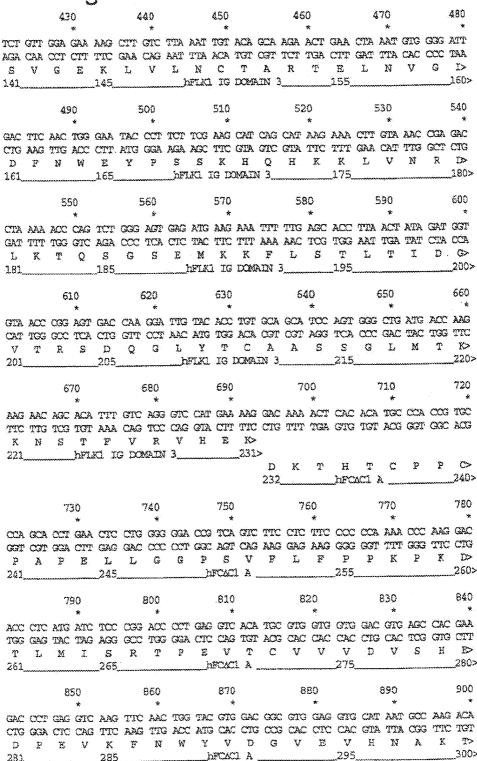
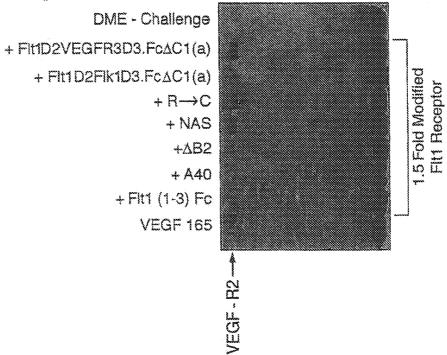


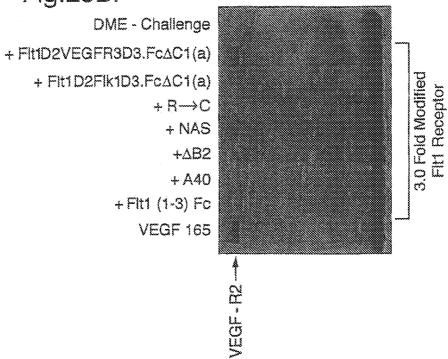
Fig.24C.

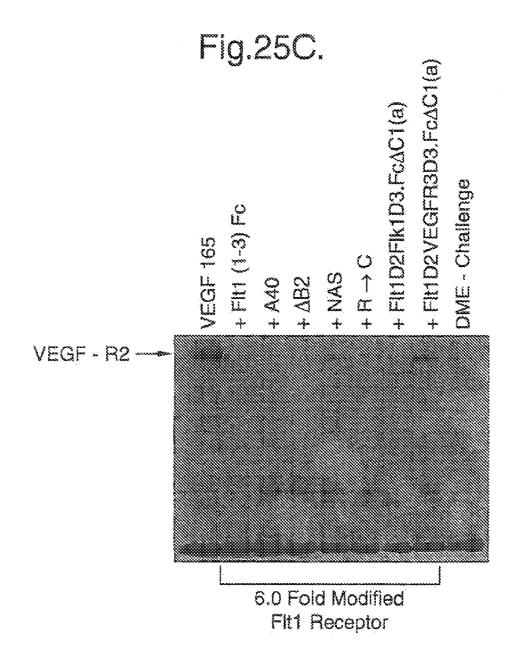
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TIT K 381	OCG G	TIC AAG F	50 X TAT ATA Y	CCC GGG P _385	AGC TCG S	160 * GAC CTG D	ATC TAG T	GCC A L	1170 * CAC V IFCAC 1230	GAG CTC E 1 A	TGG ACC W	GAG CTC E 12	AGC TCG S	: AAT N N 395	G GGG	190 CAG GTC Q 250	CCC GGC P	GAG	1200 * AAC TTG N> _400> 1260 *
TIT  K 381, AAC	CCG G TAC NTG	TIC AAG F 12 AAG	TATATATA	CCC GGG P 385	AGC TOG S	SAC CTG D 220	ATC TAG I	GCCC A A 	1170  CAC  FCAC  1230  CAC  CAC  CAC  CAC  CAC  CAC  CAC  C	GAG	TGG ACC W	GAG GTC E 12	AGC TCG S 40 * TCG AGC	: AAI TIM N _395	G TTC	190 CAG GTC Q .250 *	CCCC F F TAC	GAG E E AGG	AAC TTG N> 400>
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AAC TIG N 401	TACO	TIC AAG F 12 AAG TIC K 12 CICGC V	TATA Y 10 * ACC TGG T 70 * GACC	CCCC GGG P 385 TGC T 405 TCCC K 425	AGC TOG S  1 COTT GGA P  1 AGC TOG S	160  * GAC CTG D  220  * CCC GGS P  280  * AGS TCC R	ATC TAG	GCCCAC	1170  ** GREAC  V  IFCAC  123C  * GREAC  129C  129C  129C  CHECAC  CHECAC  129C  CHECAC  CHECAC  129C  CHECAC	GAG CTC E 1 A 1 A 2 S 5 S 11 A 2 CCC G G G 11 A	TOG ACC W GACC CIG D CIG TTG	111 GAG CTC E 122 GGGG G 13 CGGG V	AGGC S S S S S S S S S S S S S S S S S S	Y TOP	G G G G G G G G G G G G G G G G G G G	190 * CAG C GTC Q 2250 * CTC GAG L 310 * CTC C TCC S GAG S TCC S GAC S G	COOC F F TAC Y Y COCC GTC COCC V	GALCACE  E  CACC  E  CACC  E  CACC  ACC	1200  * AAC  * TTG  N>  400>  1260  * TTC  * AAG  * AAG  * TTC  * AAG  * AAG  * TTC  * AAG  *
AAC TIG N 401 CIC GAG L 421	TAC Y ACC TCC T	TIC HAGE F 122 AAGS TIC K 122 CAG V 133	TATA Y  10 * ACC TGG T  70 * GACC * G	CCCC GGGG P _385 TGCC T _405 AAGG TTCC K _425	AGC TOG S  1 CCCT GGA P  1 AGC TOG S	160  * GAC CTG D  220  * CCC GCC P  280  * AGG TCC R	ATC TAG	GCCCAC	1170  ** GREAC  V  IPCAC  123C  * GREAC  129C  129C  129C  135C  135C	GAG CTC E 1 A 1 A 2 S 5 S 11 A 2 CCC G G G C11 A	TOG ACC W GACC CIG D	111 GAG CTC E 122 GGGC G 13 CAG V	AGGC S S S S S S S S S S S S S S S S S S	YAATI NA 395 YAATI AAAA FAAAA AAAA AAAA AAAA AAAA AAAA	G G G G G G G G G G G G G G G G G G G	190 * CAG C GTC Q 250 * CTC GAG L 1310 * C TCC S ACC S 1370 *	CCCC F CCCC F CCCC V V V V V V V V V V V	GALCACE  E  C  AGE  TAGE  M  M	1200  * AAC  * TTG  N>  400>  1260  * TTC  * AAG  * AAG  * TTC  * AAG  *
AAC TIG AAC TIG AC TIG AC TIG AC AC TIG AC AC TIG AC AC TIG AC	TAC ACC TCC TCC TCC TCC TCC TCC TCC TCC	TTC AAG F 122 AAG CAC V 133	TATA Y  10 * ACC TGG T  70 * GACC * CGAC * C	CCCC GGGG P _385 ACGC TGCC TGCC TGCC K _405	AGC TOG S  1 CCT GGA P  1 AGC TOG S	AGG P P P P P P P P P P P P P P P P P P	ATC TAG I GRO CAC V	GCCCAC A A CCCCC GAC C CCAC C CCAC C CCAC C C C	1170  ** GREAT  123C  ** GRACE  D  129C  129C  129C  135C  AM  135C  AM	GAG CTC E 1 A 1 A 2 TCC S S S 11 A 1 CCC G G G C11 A	TOG ACC W GACC CIG D CIG N	111 GAG CTC E 122 GGGG GGV 13 13 13	AGGO S S S S S S S S S S S S S S S S S S	AAT N A TO	I GOOD GOOD GOOD GOOD GOOD GOOD GOOD GOO	190 * CAG C GTC Q 250 * CTC GAG L 1310 * C TCC S ACC S 1370 *	CCCC F F TAC Y Y CCCC V V AA CAA CAA CAA CAA CAA CAA CAA	GALCACCOM B COMMAND B COMM	1200  * AAC  * TTG  N>  400>  1260  * TTC  * AAG  *
AAC TIG AAC TIG AC TIG AC TIG AC TIG AC TIG AC TIG	TACO TOCO	TTCC AAG F  12 AAG TTCC K  12 CAC V	TATA Y  10 * ACC TGG T  70 * GACC CGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG	CCCC GGGG P _385 TGCC T _405 AAGG TTCC K _425	AGC TOG S  1 CCT GGA P  1 AGC TOG S  1 CGGA AGC GGGG GGGA GGGGG GGGGG GGGGGGGG	160  * GAC CTG D  * CCC * CCC * CCC * CCC R  340 * TAC	ATC TAG I GRE CAC V TGE ACC W	GCCCAC  CACC  CACC	1170  ** GREAT  V  FOAC  123C  ** GREAT  129C  129C  129C  135C  135C  TTC	GAG CTC E 1 A 1 A 2 TCC S S S S C CCC G G G C TCC C	TOG ACC W GACC CIG CIG D AACC CIG CIG CIG CIG CIG CIG CIG CIG CIG C	111 GAG CTC E 122 GGGC GGC CGG V 13 TCC TCC SGAG V	AGGO * TOO S AGGO	C TCC AGE	I GOOD G G G G G G G G G G G G G G G G G	190 * CAG	CCCC F F TAC Y Y CCCT V V AAA TTT AAA TTT AAA	GALCACCOM B COMMAND B COMM	1200  * AAC  * TTG  N>  400>  1260  * TTC  * AAG  * TTC  * AAG  * TTC  * AAG  * TTC  * AAG  * TTC  AAG

Fig.25A.

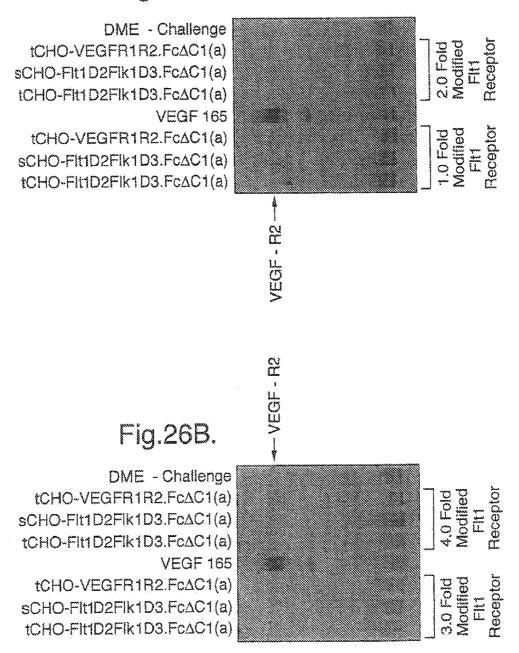


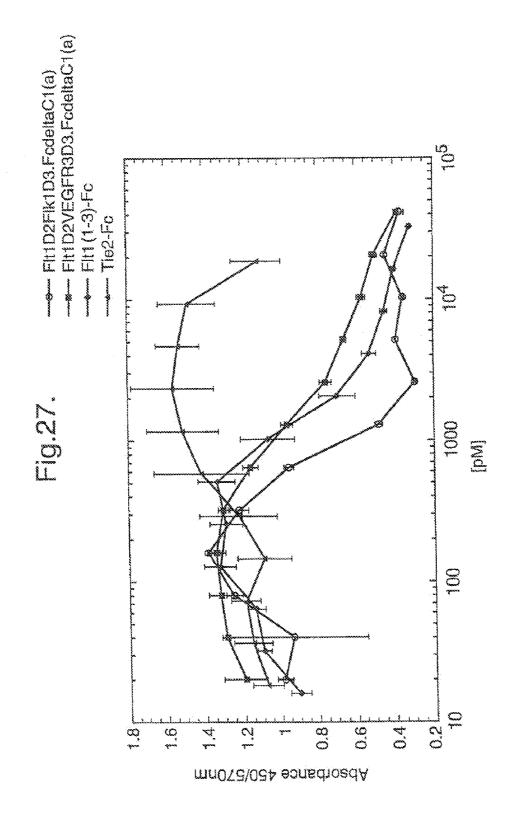
## Fig.25B.





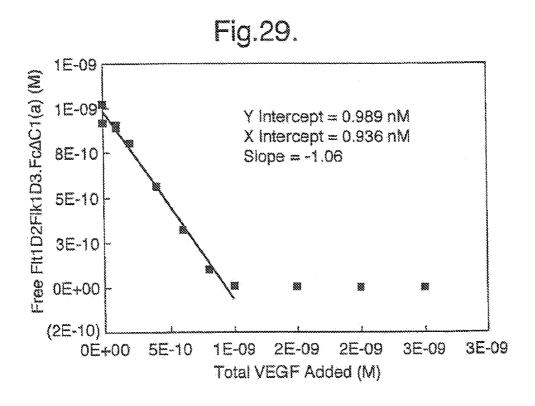
## Fig.26A.

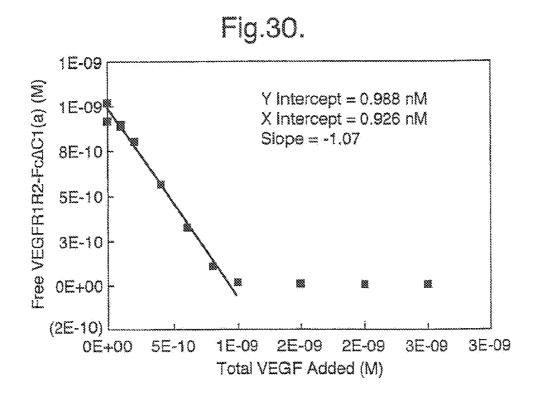


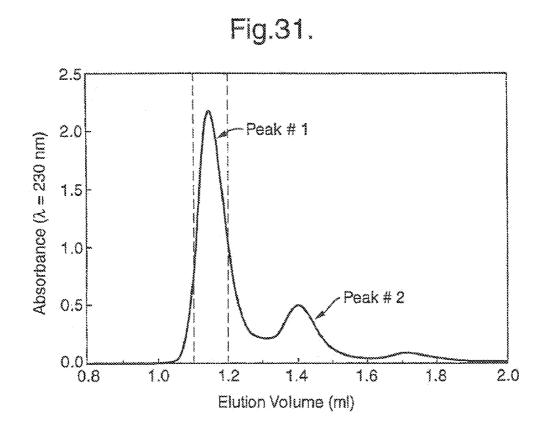


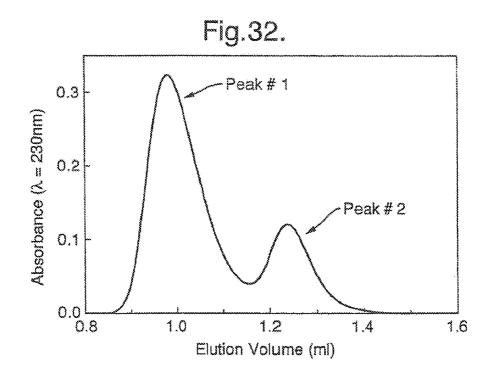
හ් ව <u>ව</u>

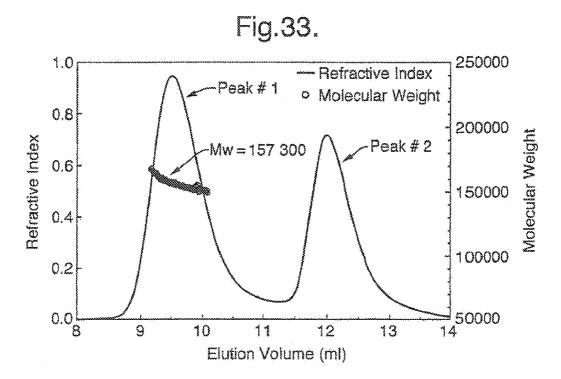
Binding Stoichio	toichiometry of hVEGF165 to FII1	metry of hVEGF165 to FIt1D2Flk1D3.FcΔC1(a) & VEGFR1R2-FcΔC1(a)
hVEGF165 (nM)	hVEGF165 (nM) VEGF/FIHD2FIKID3.FcΔC1(a)	VEGF/VEGFH1R2-FcAC1(a)
	0.93	0.98
Ç	0.97	0.94
20	que	0.99
Average ± StDev	0.96 ± 0.03	0.97 ± 0.02

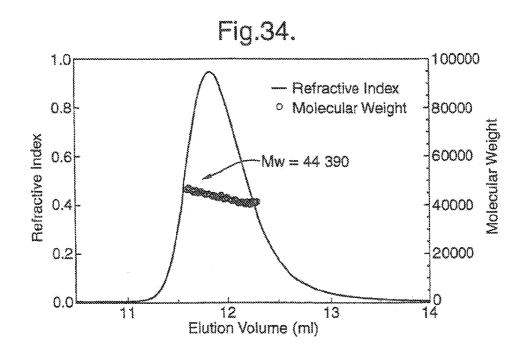


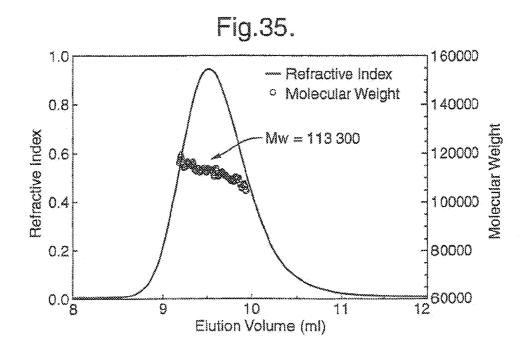








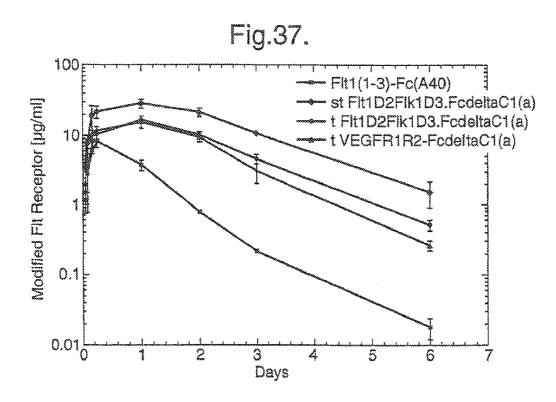


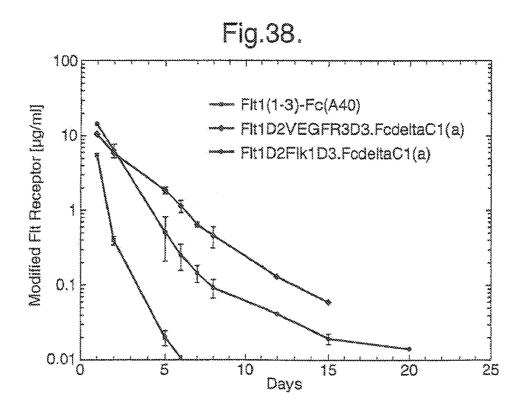


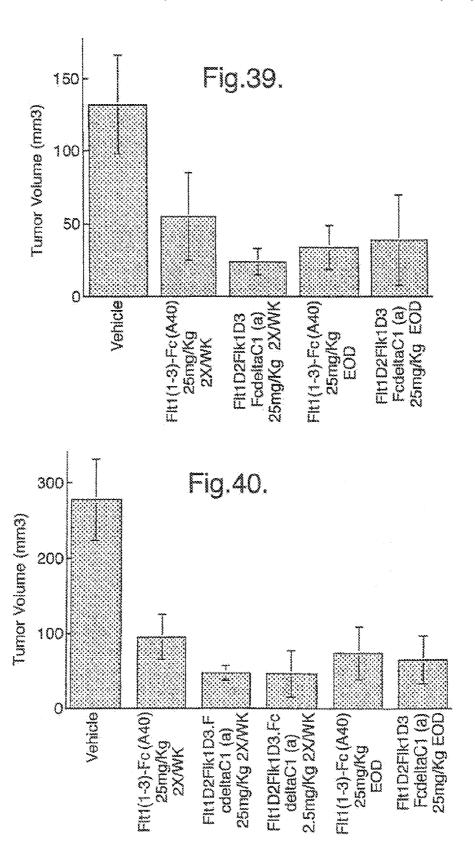
RWQQGNVFSCSVMHEALINHYTQKSLSLSPGK

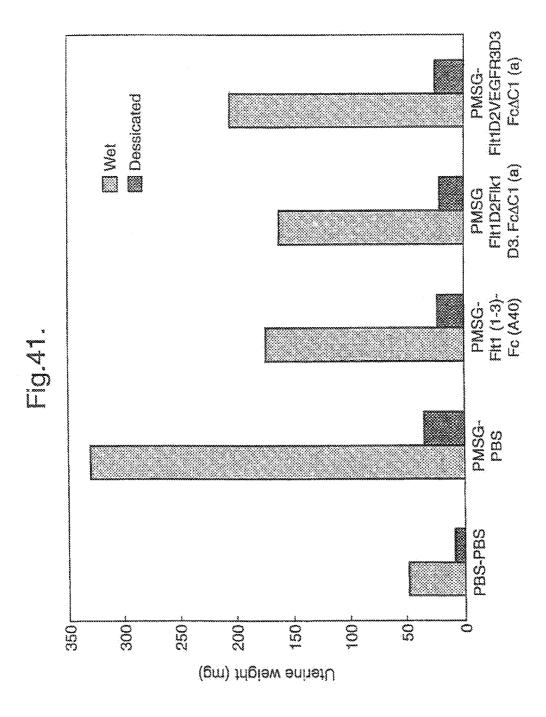
300 VSHEDPEVKFŇWÝVDGVEVHNAKTKPREEQY<u>N</u>STYRVVSVLTVLHQDWLN 30 VVL SPSHGIEL SVGEKL VLINCTARTEL NVGIDFNWEYPSSKHQHKKL VNR DI.KTOSGSEMKKFILSTLTIDGVTRSDQGLYTCAASSGLMTKKNSTFVRVH 350 GKEYK<u>C</u>KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNOVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS EKGPGDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD KRIIWDSRKGFIISNATYKEIGLLTCEATVNGIILYKTNYLTHROTNITII GRPFVEMYSEIPEIHEMTEGRELVIPQRVTSP<u>M</u>ITVILKKFPLDILIPDG

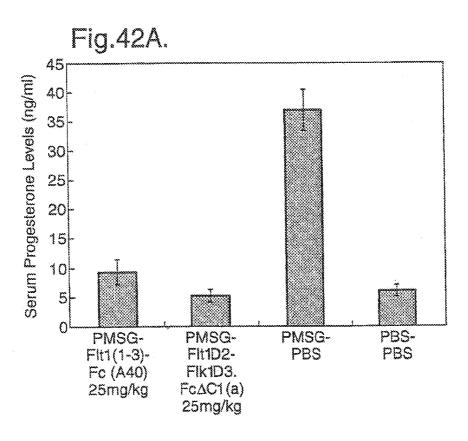
> Mylan Exhibit 1161 Mylan v. Regeneron, IPR2021-00881 Page 53

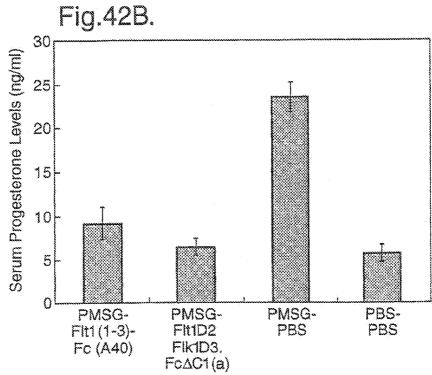












# CELL CULTURE COMPOSITIONS CAPABLE OF PRODUCING A VEGF-BINDING FUSION POLYPEPTIDE

This application is a continuation of U.S. patent application Ser. No. 12/334,927, filed Dec. 15, 2008, now U.S. Pat. No. 7,964,377, which is a continuation of U.S. patent application Ser. No. 12/102,648, filed Apr. 14, 2008, now U.S. Pat. No. 7,524,499, which is a divisional 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, 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 modified in such away 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 mammal.

#### BACKGROUND

The ability of polypeptide ligands to bind to cells and thereby elicit a phenotypic response such as cell growth, 35 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 generally the most distinctive portion of the molecule, as it provides 40 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 intracellular domain. The particular array of sequence motifs 4 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 catalytic intracellular domains include the receptor tyrosine 50 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. 25:1454-1466) which is also generally limited to the cells of the nervous system, G-protein coupled receptors such as the β<sub>2</sub>-adrenergic receptor found on, for instance, cardiac muscle cells, and the multimeric IgE high affinity receptor Fc∈RI which is localized, for the most part, on mast cells and basophils (Sutton & 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 61:203-212; Schlessinger & Ullrich, 1992, Neuron 9:383-65 391) and molecular interactions between dimerizing intracellular domains lead to activation of catalytic function. In

2

some instances, such platelet-derived, growth factor (PDGF), the ligand is a dimer that binds two receptor molecules (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 FceRI receptor ligand, IgE, exists bound to FceRI in a monomeric fashion and only becomes activated when antigen binds to the IgE/FceRI 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 20 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 neuronsons the brain and periphery (Lindsay, R. M, 1993, in Neurotrophic Factors, S. E. Loughlin & J. H. Fallon, eds., pp. 257-1284, San Diego, Calif., Academic Press). Fc∈RI is localized to a very limited number of types of cells such as mast cells and basophilis. Mast cells derive from bone marrow pluripotent hematopoietic stem cell lineage, but complete there 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 neurorophin 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 60 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. at al., 1991 Oncogene 6, pp. 1677-1683; Terman, B. I. et al., 1992 Biochem. Biophys. Res. 5 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 10 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 15 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 20 inflammation is the plasma leakage that results from intercellular gaps that form in the endothelium olvenules. Most experimental models of inflammation indicate that these intercellular gaps occur between the endothelial cells of post-Pathol., 1998, 152:1463-76). It has been shown that certain lectins may be used to reveal features of focal sites of plasma 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 30 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 subenof plasma leakage (Thurston, G., et al., Am J Physiol., 1996; 271: H2547-62).

The properties of the microvessels are, dynamic. Chronic inflammatory diseases, for example, are associated with microvascular remodeling, including angiogenesis and 40 microvessel enlargement. Microvessels can also remodel by 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 45 increased immunoreactivity for P-selectin. In addition, these 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 50 vascular permeability and/or plasma leakage. For example, 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 55 formoterol reduces microvascular leakage by inhibiting 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 60 thought to be largely specific for vascular endothelial, cells. 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

U.S. Pat. No. 6,011,003, issued Jan. 4, 2000, in the name of 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 vascularendothelial 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 protein's bind to VEGF and antagonize the endothelial cell proliferative and angiogenic activity

PCT Publication No. WO 97/13787, published Apr. 17, capillary and collecting venules (Baluk, P., et al., Am. J. 25 1997, in the name of Toa 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. dothelial vessel wall exposed by gaps that correspond to sites 35 42:242-249, disclose that because monoclonal antibodies (MAbs) are basic, positively charged proteins, and mammalian 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

> 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. Pharmatokinet. 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 dele-

tions 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 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 15 receptor component is the only VEGF receptor component of 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 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 25 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 30 VEGF receptor is upstream of than nucleotide sequence encoding 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 or 35 the first VEGF receptor is downstream of the nucleotide a sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

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

In another embodiment, the immunoglobulin domain is selected from the group consisting of the Fc domain of 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 modi- 45 fied Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists of a nucleotide sequence selected from the group consisting of (a) the nucleotide sequence set forth in FIG. 13A-13D (SEQ ID No:3); (b) the nucleotide sequence set forth in FIG. 14A-14C (SEQ ID 50 NO:5); (c) the nucleotide sequence set forth in FIG. 15A-15C (SEQ ID NO:7); (d) the nucleotide sequence set forth in FIG. 16A-16D (SEQ ID NO:9); (e) the nucleotide sequence set forth in FIG. 21A-21C (SEQ ID NO:11); (f) the nucleotide sequence set forth in FIG. 22A-22C (SEQ ID NO:13); (g) the 55 nucleotide sequence set forth in FIG. 24A-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 60 activity or the modified Flt1 receptor 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 binding a VEGF molecule to form a nonfunctional complex comprising a multimer of the fusion polypeptide. 6

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 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 area 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 FIG. 10A-10D (SEQ ID NO:1) or FIG. 24A-24C (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 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 spe-40 cifically 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 Flk1.

In yet a further embodiment of the fusion polypeptide, the second VEGF receptor 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 10 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 <sup>15</sup> 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 comprising 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 FIG. 13A-13D (SEQ ID NO:4); (b) the amino acid sequence set forth in FIG. 14A-14C (SEQ ID NO:6); (c) the amino acid sequence set forth in FIG. 15A-15C (SEQ ID NO:8); (d) the amino acid sequence set forth in FIG. 16A-16D (SEQ ID NO:10); (e) the amino acid sequence set forth in FIG. 21A-21C (SEQ ID NO:12); (f) the amino acid sequence set forth in FIG. 22A-22C (SEQ ID NO:14); and (g) the amino acid sequence set forth in FIG. 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 inhib- 35 iting 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 Flt1(1-3)-Fc is able to enter the gel and equilibrate at pI 52.

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 acetylated 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 BIACORETM-based assay. Acetylated (columns 13-16), pegylated (columns 17-20), and heparin-treated Flt1(1-3)-Fc (columns 21-24) are each able to completely compete with the BIA-CORETM 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 BIACORETM chip-bound Flt1 (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.

FIG. **4**. Binding of unmodified Flt1(1-3)-Fc, acetylated 65 Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc, and Fc to VEGF in an ELISA-based assay. Both pegylated and acetylated Flt1

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(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 µg/ml; acetylated: 1.5 µg/ml-4.0 µg/ml; and pegylated: approximately 5 µg/ml.

FIG. 6A-6B. IEF gel analysis of unmodified and step-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 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 step-acetylated Flt1(1-3)-Fc proteins to MATRIGEL® coated plates. As with the irrelevant control protein, rTie2-Fc, step-acetylated 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 to extracellular matrix components.

FIG. **8**. Binding of unmodified Flt1(1-3)-Fc and stepacetylated Flt1(1-3)-Fc in a BIACORE<sup>TM</sup>-bated assay. 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, the both unmodified and step-acetylated Flt1(1-3)-Fc are better able to compete for VEGF binding, but there is still insufficient Flt1(13)-Ft protein (either unmodified or step-acetylated) to completely saturate the available VEGF. However, at 5.0 μg/ml which is several times greater a 1:1 stoichiometric ratio, both the Flt1(1-3)-Fc and the step-acetylated Flt1(1-3)-Fc proteins are able to saturated 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/mice (23-28 g) were injected 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  $C_{max}$  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.

FIG. 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

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

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

FIG. 14A-14C. Nucleic acid (SEQ ID NO:5) and deduced amino acid sequence (SEQ ID NO:6) of Mut2: Flt1(2-3<sub>AB</sub>)-

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

FIG. 16A-16D. Nucleic acid (SEQ ID NO:9) and deduced amino acid sequence (SEQ ID NO:10) of Mut4: Flt1(1-

 $3_{R\to N}$ )-Fc. FIG. 17. Binding, of unmodified Flt1(1-3)-Fc, bait region deletion mutant Flt1(1-3)-Fc, and Flt1(1-3)<sub> $R\to N$ </sub> mutant pro- 10 teins 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 BIACORETM chip. At 15 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 BIACORETM chip. At 1.0 µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, which is 20 approximately a 10:1 stoichiometric ratio, the Flt1(1-3)-Fc proteins are able to block binding of VEGF to the BIA-CORE™ chip, but they are not equivalent. Unmodified, acetylated, and Mut1: Flt1(1-3 $_{\Delta B}$ )-Fc are essentially equal in  $3_{R \to N}$ )-Fc is somewhat less efficient at blocking binding.

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

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

FIG. 20. Pharmacokinetic profiles of unmodified Flt1(1-3)-Fe, Mut1: Flt1-3 $_{\Delta B}$ )-Fe, Mut2; Flt1(2-3 $_{\Delta B}$ )-Fe, and Flt1(2-3) mutant proteins, the  $C_{max}$  for these reagents was as follows: 45 Unmodified Flt1(1-3)-Fc-0.15 μg/ml; 40 fold molar excess acetylated Flt1(1-3)-Fc-1.5  $\mu$ g/ml; and Mut1: Flt1(1-3<sub>A3</sub>)-Fc-

FIG. 21A-21C. Nucleotide (SEQ ID NO:11) and deduced amino acid sequence (SEQ ID NO:12) of the modified Flt1 50 receptor termed Flt1D2.Flk1D3.FcΔC1(a).

FIG. 22A-22C. Nucleotide (SEQ ID NO:13) and deduced amino acid sequence (SEQ ID NO:14) of the modified Flt1 receptor termed Flt1D2.VEGFR3D3.FcΔC1(a).

FIG. 23. Extracellular Matrix (ECM) Assay. The results of 55 this assay demonstrate that the Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) proteins are considerably less sticky to the ECM as compared to the Flt1(1-3)-Fc protein.

FIG. 24A-24C. Nucleotide (SEQ ID NO:15) and deduced amino acid sequence (SEQ ID NO:16) of the modified Flt1 60 receptor termed VEGFR1R2-FcΔC1(a).

FIG. 25A-25C. Phosphorylation assay. At a 1.5 molar excess of either Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3.FcΔC1(a) there is complete blockage of receptor stimulation by these three modified Flt1 receptors as 65 compared to control media challenge. In contrast, transient Flt1D2VEGFR3D3.FcΔC1(a) does not show significant

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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 cellsurface receptors.

FIG. 26A-26B. Phosphorylation assay. Detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows 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 4 fold molar excess (FIG. 26B) of either transient Flt1D2Flk1D3.FcΔC1(a), stable Flt1D2Flk1D3.FcΔC1(a), or transient VEGFR1R2-FcΔC1 (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.FcΔC1 (a) and Flt1D2.VEGFR3D3.FcΔC1(a), plus an irrelevant their ability to block VEGF binding, whereas Mut4: Flt1(1- 25 receptor learned Tie2-Fc as a negative control, were titrated from 40 nM to 20 pM 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 VEGF165induced cell proliferation at any concentration whereas Flt1D2.Flk1D3.FcΔC1(a) blocks 1.56 nM VEGF165 with a half maximal dose of 0.8 nM. Flt1(1-3)-Fc and Flt1D2VEGFR3D3FcΔC1(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 unit 0.38 absorbance units.

> FIG. 28. BIACORE™ analysis of Binding Stoichiometry. Binding Stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1D2Flk1D3.FcΔC1 (a) or VEGFR1R2-Fc $\Delta$ C1(a), listing 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 $\Delta$ 1(a) molecule.

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

> FIG. 31. Size Exclusion Chromatography (SEC) under Peak native conditions. #1 represents Flt1D2Flk1D3.FcΔC1 (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-

 $OSE^{TM}$  12 PC.3.2/30 equilibrated in 6 M GuHCl and eluted. Peak #1 represents Flt1D2Flk1D3.Fc $\Delta$ C1(a) and peak #2 represents VEGF165.

FIGS. 33-35. 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. 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 receptorligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.FcΔC1(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 the mW of R1R2 at the peak is 113 300 (FIG. 35).

FIG. 36. Peptide mapping and glycosylation analysis. The structures and glycosylation sites Flt1D2.Flk1D3.FcΔC1(6) (SEQ ID NO:12) were determined by a peptide mapping method. There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a), six of them belong to the 25 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 disulfide bonded to Cys306. Cys352 is disulfide bonded to Cys410. There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.Fc∆C1(a) (SEQ ID NO:12) and are found to be glycosylated to varying degrees. Complete glycosylation is observed at Asn33, Asn193, and Asn282. Partial glycosylation is observed on Atn65 and Atn120. Sites of glycosylation are highlighted by underline in the figure.

FIG. 37. Pharmacokinetics of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1 (a). Balb/c mice were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a), CHO stably Flt1D2.Flk1D3.FcΔC1(a), and CHO transiently expressed VEGFR1R2-Fc $\Delta$ C1(a). The mice were tail bled at 1, 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 Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a). The 50  $T_{max}$  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 transient VEGFR1R2-Fc $\Delta$ C1(a) was 24 hrs. The C $_{max}$ Flt1(1-3)-(A40) was 8 µg/ml. For both transients (Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)) the 55  $C_{max}$  was 18 µg/ml and the  $C_{max}$  for the stable VEGFR1R2-Fc $\Delta$ C1(a) was 30  $\mu$ g/ml.

FIG. 38. Pharmacokinetics of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1 (a). Balb/c mice were injected subcutaneoutly with 4 mg/kg 60 of Flt1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a) and CHO transiently expressed Flt1D2.VEGFR3D3.FcΔC1(a). The mice were tail bled at 1, 2, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc, 65 Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1 (a). Flt1(1-3)-Fc (A40) could no longer be detected in the

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serum after day 5 whereas Flt1D2.Flk1D3.Fc $\Delta$ 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.Fc $\Delta$ C1(a) at 25 mg/Kg significantly decreases the growth of subcutaneous HT-1080 fibrosarcoma tumors.

FIG. 40. The Ability of Flt1D2.Flk1D3.Fc $\Delta$ C1(a) to inhibit C6 Glioma Tumor Growth In Vivo. Every other day or 2 times a week treatment of SCID mice with Flt1D2.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), Flt1D2.Flk1D3.FcΔC1(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.

FIGS. **42**A-**42**B. 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 Flt1D3.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 receptor based VEGF antagonist that has a pharmacokinetic 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 receptor-based 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 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 apolar amino acids (i.e. leucine, valine, isoleucine,

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and phenylalanine) comprise the transmembrane domain. The extracellular domain comprises the amino acids that 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. 5 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 10 have become available to provide protein chemists with information about making predictions about protein domains.

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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 15 chimeric polypeptide molecules when introduced into an appropriate host cell. Appropriate host cells include, but are 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 20 be used to construct expression vectors encoding the chimeric polypeptide molecules under control of transcriptional/translational control signals. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination) (See Sambrook, et 25 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 chi- 30 meric 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 DNA molecule. For example, expression of the chimeric polypeptide molecules described herein may be controlled by 35 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 terminal repeat as described in Squinto et al., (1991, Cell 65:1-20); the SV40 early promoter region (Bernoist and 40 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 al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 45 78:144-1445), the regulatory sequences of the metallothionine gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, 50 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 fungi such as the Gal 4 promoter, the ADH (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, 55 alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold 60 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

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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. 1:268-270), 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 bated 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. 5,888,304). 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 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 agonists 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 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; generalized edema associated with burns; ascites and pleural effusion associated with tumors, inflammation or trauma; othronic airway inflammation; capillary leak syndrome; sepsis; kidney disease associated with increased leakage of protein; and eye disorders such as age related macular degeneration and diabetic retinopathy.

An amino acid sequence analysis of Flt1(1-3)-Fc revealed 35 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 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 when injected into mice. In order to test this hypothesis, Flt1(1-3)-Fc protein was acetylated at the lysine residues to 45 reduce the basic charge. Acetylated Flt1(1-3)-Fc was then tested in the assays described infra.

The following examples are offered by way of illustration 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., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., 60 Wiley-Interscience, NY), 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 (Gaithersburg, Md.). The transfected CHO K1 cells were grown in glutamine-free DMEM (JRH, Kansas City, Mo.) containing

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 $25~\mu M$  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  $55~\mu g/cell/day$ .

The selected clone was expanded in 225 cm<sup>2</sup> 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 glutamine free ISCHO medium (Irvine Scientific, Santa Ana, Calif.) containing 5% fetal bovine serum (FBS from HYCLONETM 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 0.3×10<sup>6</sup> cells/mL. After the cells reached a density of 3.6×10<sup>6</sup>/mL and were adapted to suspension they were transferred to a 60 L bioreactor (ABEC, Allentown, Pa.) at a density of 0.5×10<sup>6</sup> 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 3.1×10<sup>6</sup> 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, N.J. with the exception of PBS, which was obtained as a 10× concentrate from Life Technologies, Gaithersburg, Md. Protein A Fast Flow and SUPER50 DEX™ 200 preparation grade resins were obtained from Pharmacia, Piscataway, N.J. Equipment and membranes for protein concentration were obtained from Millipore, Bedford, Mass.

Approximately 40 L of 0.45 µm-filtered CHO conditioned 55 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 Na<sub>2</sub>HPO<sub>4</sub>. 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 SUPERDEX<sup>TM</sup> 200 preparation grade resin (10 cm×55 cm) and run in PBS con-

taining 5% glycerol. The main peak fractions were pooled, sterile filtered, aliquoted and stored at -80° C.

#### Example 3

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

Two milligrams of Flt1(1-3)-Fc protein were acetylated as 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 Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc were analyzed by Standard IEF analysis. As shown in FIG. 1, 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 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 matrix components. In this assay, 96-well tissue culture plates are coated with 30 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 Flt1(1-3)-Fc, or rTie2-Fc (an irrelevant control) protein are added to the wells. 35 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 the wells. Finally, alkaline phosphatase substrate is added to the wells 40 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 (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 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 counterintuitive 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 by altering the positive charge or by decreasing the pI of 65 Flt1(1-3)-Fc, but rather by physically shielding the positive charges from interacting with the extracellular matrix. Appli-

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cants decided to attempt to improve the pharmacokinetic properties of Flt1(1-3)-Fc molecules by attaching strands of 20K PEGs as described infra.

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.; SUPEROSE<sup>TM</sup> 6 column from Pharmacia, Piscataway, N.J.; PBS as a 10× 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 terminal 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 reaction's 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 BIACORE<sup>TM</sup>-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 µg/ml VEGF and either unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc or pegylated Flt1(1-3)-Fc (each at 25 µg/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 at 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 BIACORETM chip-bound Flt1(1-3)-Fc for VEGF binding. However, washing the bound samples with 0.5 M NaCl (columns 7-8) resulted in a binding profile similar to the modified

forms of Flt1(1-3)-Fc, indicating that the unmodified proteins 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 modifying 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 pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, acetylated Flt1  $\,^{25}$ (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 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: 0.06 μ/ml-0.15 μg/ml; acetylated: 1.5 μg/ml-4.0 μg/ml; and pegylated: approximately 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 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 manual provided with the sulfo-NHS-Acetate modification 55 kit (Pierce Chemical Co., Rockford, Ill., Cat. #26777).

#### Example 10

#### Characterization of Step-Acetylated Flt1(13)-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 FIG. **6**A-**6**B, unmodified Flt1(1-3)-Fc protein was not able to migrate into the gel due to its extremely 65 high pI (greater than 9.3). However, most of the step-acetylated Flt1(1-3)-Fc samples (30-100 fold molar excess

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samples) were able to migrate into the gel and equilibrate at pls 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 accomplished by adding a secondary alkaline phosphataseconjugated 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 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 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 μg/ml, which is several times greater than a 1:1 stoichiometric 60 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 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:  $0.06 \mu g/ml$ ; 10 fold molar excess sample: -0.7μg/ml, 20 fold molar excess sample—2 μg/ml, 40 fold molar excess sample—4 µg/ml, 60 fold molar excess sample—2  $\mu g/ml$ , 100 fold molar excess sample—1  $\mu g/ml$ . 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 $_{\Delta B}$ )-Fc

Based on the observation that acetylated Flt1(1-3)-Fc, which has a pI below 6, has much better pharmacokinetics 25 than the highly positive unmodified Flt1(1-3)-Fc (pI>9.3), it was asked whether the difference in pharmacokinetics could 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 sur- 30 face of the Flt1(1-3)-Fc protein that constituted specific binding sites for extracellular matrix components. For example, 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 35 protein; some of the literature has identified "consensus sequences" for such heparin binding sites (see for example 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 40 the residues come from different regions of the primary sequence and are only brought together when the protein 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 45 there is a cluster of positively charged amino acid residues in the primary sequence, it is not unreasonable to surmise that 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 vari- 50 ous domains have been described (see for example Tanaka et al., 1997, Jpn. J. Cancer Res. 88:867-876). Referring to the nucleic acid and amino acid sequence set forth in FIG. 10A-10D of this application, one can identify the signal sequence for secretion which is located at the beginning of the sequence 55 and extends to the glycine coded for by nucleotides 76-78. The mature protein begins with Ser-Lys-Leu-Lys, 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 60 394 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 65 (nucleotides 1006-1701 or amino acids. Glu-Pro-Lys to Pro-Gly-Lys-stop).

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A more detailed analysis of the Flt1 amino acid sequence reveals that there is a cluster, namely, amino acid residues 272-281 (KNKRASVRR) of FIG. 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 (FIG. 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 10 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 20 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 FIG. 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

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 FIG. **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  $(2-3_{\Delta B})$ -Fc, was derived from the Mut1: Flt1 $(1-3_{\Delta B}$ -Fc construct by deletion of Flt1 Ig domain 1 encoded by nucleotides 79-393 (see FIG. 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 (Genetic's 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<sub>AB</sub>)-Fc is set forth in FIG. 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 and the final construct was sequence-verified as described supra. The sequence of Mut3: Flt1(2-3)-Fc is set forth in FIG. 5 15A-15C.

#### Example 14

Construction of Flt1-3)-Fc Basic Region N-glycosylation Mutant Designated Mut4: Flt1(1-3<sub> $R\rightarrow N$ </sub>)-Fc

A final construct was made in which a N-glycosylation site was introduced into the middle of the basic region of Flt1 Ig domain 3. This construct was designated Mut4: Flt1(1- $3_{R \to N}$ )-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 FIG. 10A-10D). The resulting amino acid sequence is therefore changed from Arg-Ala-Ser to Asn-Ala-Ser; which matches the canonical 20 signal (Asn-Xxx-Ser/Thr) for the addition of a N-glycosylation site at the Asn residue. The sequence of Mut4: Flt1(1- $3_{R\to N}$ )-Fc is set forth in FIG. **16**A-**16**D.

#### Example 15

Characterization of Acetylated Flt1(1-3)-Fc, Mut1: Flt1(1-3<sub> $\Delta B$ </sub>)-Fc, and Mut4: Flt1(1-3<sub> $R \rightarrow N$ </sub>)-Fc Mutants

Binding to extracellular matrix components. To determine 30 whether the three modified proteins were more or less likely to have improved pharmacokinetic properties, MATRIGEL® coated 96-well dishes (as described supra) were incubated with varying concentrations of the mutant proteins and detected with anti-human Fc/alkaline-phosphatase conju- 35 gated 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)-Fc protein bound somewhat more weakly, the Mut1: Flt1(1-3 $_{\Delta B}$ )-Fc protein bound more weakly still, and the Mut2:  $Flt1(2-3_{\Delta B})$ -Fc 40 protein showed the best profile, binding more weakly than any of the other mutant proteins. The Mut4:  $Flt1(1-3_{R\rightarrow N})$ -Fc glycosylation mutant protein showed only marginal benefit on the MATRIGEL® assay. These results confirm the hypothesis that a linear sequence of positive amino acids can 45 be deleted from the primary sequence resulting in a decrease in charge interaction with extracellular matrix components.

Binding of Mut1: Flt1(1-3<sub> $\Delta B$ </sub>)-Fc and Mut4: Flt1(1-3<sub> $R \rightarrow N$ </sub>)-Fc in a BIACORETM-Based Assay. Unmodified and acetylated Flt1(1-3)-Fc and genetically modified Mut1: Flt1(1- 50 3)-Fc—0.15  $\mu$ g/ml; 40 fold molar excess acetylated Flt1(1- 50 3)-Fc  $3_{\Delta B}$ )-Fc and Mut4: Flt1(1- $3_{R\to N}$ )-Fc proteins where 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 (0.25, 0.5, or 1.0 µg/ml) was immobilized on the surface of a BIACORETM chip (see BIACORETM Instruction 55 Manual, Pharmacia, Inc., Piscataway, N.J., for standard procedures) and a solution containing 0.1 µg/ml VEGF and either purified or COS cell supernatant containing unmodified Flt1 (1-3)-Fc (at approximately  $(0.25, 0.5, \text{ or } 1.0 \,\mu\text{g/ml})$ , purified acetylated Flt1(1-3)-Fc (at  $(0.25, 0.5, \text{ or } 1.0 \,\mu\text{g/ml})$ , COS cell 60 supernatant containing Mut1; Flt1(1-3<sub>AB</sub>)-Fc (at approximately (0.25, 0.5, or 1.0  $\mu g/ml$ ), or COS cell supernatant containing Mut4 Flt1(1-3 $_{R\rightarrow N}$ )-Fc (at approximately (0.25, 0.5, or 1.0  $\mu$ g/ml) were passed over the Flt1(1-3)-Fc coated chip. As shown in FIG. 17, at the sub-stoichiometric ratio 65 (0.25 μg/ml Flt1(1-3)-Fc of unmodified, acetylated or genetically modified samples vs. 0.1 µg/ml VEGF), there is insuf-

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ficient 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<sup>TM</sup> 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 BIACORE  $^{\text{TM}}$  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<sub> $R\rightarrow N$ </sub>)-Fc is somewhat less efficient blocking binding. These results confirm the hypothesis that it is possible to reduce the nonspecific binding of a positively charged molecule by genetically removing a linear sequence of predominantly negatively charged amino acids.

Binding of Mut1: Flt1(1-3<sub> $\Delta B$ </sub>)-Fc, Mut2: Flt1(2-3<sub> $\Delta B$ </sub>)-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 <sup>25</sup> 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, Mutt Flt1(1-3 $_{\Delta B}$ )-Fc, and Unmodified Flt1(1-3)-

In vivo experiments were designed to assess the pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1- $3_{AB}$ )-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 $_{\Lambda B}$ )-Fc proteins (4 mice each). These mice were tail bled at  $1, \overline{2}, 4, 6$ , 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<sub>max</sub> for these reagents was as follows: Unmodified Flt1(1-3)-Fc—1.5  $\mu$ g/ml; and Mut1: Flt1(1-3 $_{\Delta B}$ )-Fc—0.7  $\mu$ g/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 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 (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 netcharge of +11) is not essential for binding, but confers higher affinity for VEGF than the second 14 domain, so instead of deleting it entirely, it was replaced with the equivalent domains of the Flt1 receptor relatives Flk1 (also known as VEGFR2) and Flt4 (also known as VEGFR3). chimeric molecules (denoted R1R2 These (Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)) and R1R3 (Flt1D2.VEGFR3D3-FcΔC1(a) and VEGFR1R3-FcΔC1(a)) respectively, wherein R1 and Flt1D2=Ig domain 2 of Flt1 (VEGFR1); R2 and Flk1D3=Ig domain 3 of Flk1 (VEGFR2); and R3 and VEGFR3D3=Ig domain 3 of Flt4 20 (VEGFR3)) were much less sticky to ECM, as judged by an 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 25 endothelial cells as described infra.

Construction plasmid of expression pFlt1D2.Flk1D3.FcΔC1(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 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
                                  (SEQ ID NO: 18)
(5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3')
3': Flt1D2-Flk1D3.as
                                  (SEQ ID NO: 19) 45
(5'-CGGACTCAGAACCACATCTATGATTGTATTGGT-3'
```

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 FIG. 21A-21C). The 3' 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 fusion point defined as TIID (SEQ ID NO:37) of Flt1 (corresponding to amino acids 123-126 of FIG. 21A-21C) an continuing into VVLS (SEQ ID NO:38) (corresponding to applied to appl ing to amino acids 127-130 of FIG. 21A-21C) of Flk1.

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

```
5': Flt1D2-Flk1D3.s
                                  (SEO ID NO: 21)
(5'-ACAATCATAGATGTGGTTCTGAGTCCGTCTCATGG-3')
3': Flk1D3/apa/srf.as
                                  (SEQ ID NO: 22) 65
(5'-GATAATGCCCGGGCCCTTTTCATGGACCCTGACAAATG-3'
```

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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 FIG. 21A-21C), 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 FIG. 21A-21C.

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/ΔB2.Fc, to create the plasmid pMT21/Flt1D2.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-FcΔC1(a) to produce the plasmid pFlt1D2.Flk1D3.Fc $\Delta$ C1(e). The complete DNA and deduced amino acid sequences of the Flt1D2.Flk1D3.FcΔC1(a) chimeric molecule is set forth in FIG. 21A-21C.

Construction of the expression plasmid the respective Ig domains followed by further rounds of PCR  $^{35}$  pFlt1D2VEGFR3D3Fc $\Delta$ C1(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 40 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 FIG. 22A-22C). 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 FIG. 22A-22C) and continuing into IQLL (SEQ ID NO:26) of VEGFR3 (Corresponding to amino acids 127-130 of FIG. 22A-22C).

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)
(ATTTCATGCACAATGACCTCGGTGCTCTCCCGAAATCG)

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 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.VEGPR3D3.s

(SEQ 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 FIG. 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 FIG. 22A-22C.

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 amplification primers bsp/flt1D2 and VEGFR3D3/srf as 40 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 vector pMT21/Flt1ΔB2.Fc (described supra), to create the 45 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 enzyme EcoRI and SrfI and the resulting 693 bp fragment was subcloned into the EcoRI to SrfI restriction 50 sites of the plasmid pFlt1(1-3) $\Delta$ B2-Fc $\Delta$ C1(a) to produce the plasmid designated pFlt1D2.VEGFR3D3.FCΔC1(a). The complete DNA deduced amino acid sequence of the Flt1D2.VEGFR3D3. EcΔC1(a) chimeric molecule is set forth in FIG. 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),  $100 \, \text{U}$  penicillin,  $100 \, \text{U}$  streptomycin, and 10% BCS for at least 1 hr before adding samples. The plates were then incubated for 1 hr at room temperature with varying concentrations of Flt1D2.Flk1D3.FC $\Delta$ C1(a) and Flt1D2 VEGFR3D3.Fc $\Delta$ C1(a) starting at 10 nM with subsequent

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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 anti-body (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 shown in FIG. 23 and demonstrate that the Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1 (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.Fc $\Delta$ C1(a) in CHO-K1 (E1A) Cells

A large scale (2 L) culture of *E. coli* DH10B cells carrying
the pFlt1D2.Flk1D3.FcΔC1(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 following 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 enzyme sEcoRI
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 4×10<sup>6</sup> cells/plate. Plating media was Gibco Ham's F-12 supplemented with 10% HYCLONE™ 35 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 μg of the pFlt1D2.Flk1D3.FcΔC1 (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% CO<sub>2</sub> 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 constructed by insertion of DNA encoding amino acids. SDT (corresponding to amino acids 27-29 of FIG. **24**A-**24**C) between Flt1d2-Flk1d3-Fc $\Delta$ C1(a) amino acids 26 and 27 of FIG. **21**A-**21**C (GG) and removal of DNA encoding amino acids GPG corresponding to amino acids 229-231. 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 $\Delta$ C1(a) chimeric molecule is set forth in FIG. 24A-24C.

#### Example 21

#### Cell Culture Process Used to Produce Modified Flt1 Receptors

Cell Culture Process Used to Produce 10 Flt1D2.Flk1D3.FcΔC1(a). The process for production of Flt1D2.Flk1D3.FcΔC1(a) protein using the expression plasmid pFlt1D2.Flk1D3.FcΔC1(a) described supra in Example 1 involves suspension culture of recombinant Chinese hamster ovary (CHO K1/E1A) cells with 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 detail below.

Cell Expansion. Two confluent T-225 cm² flasks containing the Flt1D2.Flk1D3.Fc $\Delta$ C1(a) expressing cell line were expanded by passaging cells into eight T-225 cm² flasks in medium (GMEM+10% serum, GIBCO) and incubated at 37° C. and 5% CO $_2$ . 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 resuspended in fresh medium then transferred to eight 850 cm² roller bottles and incubated at 37° C. and 5% CO $_2$  until confluent.

Suspension Culture in Bioreactors. Cells grown in, roller bottles were trypsinized to detach 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 35 culture. The suspension culture medium was a glutamine-free low glucose modification of 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 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° C. When a density of  $4\times10^6$  cells/mL 45 was reached the cells were transferred to a 40 L bioreactor containing the same medium and setpoints for controlling the bioreactor. The temperature setpoint was reduced to 34° C. to slow cell growth and increase the relative rate of protein expression.

Cell Culture. Process Used to Produce Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a). The same methodologies as described supra for Flt1D2.Flk1D3.Fc $\Delta$ C1(a) were used to produce Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a).

#### Example 22

#### Harvest and Purification of Modified Flt1 Receptors

Harvest and Purification of Flt1D2.Flk1D3.FcΔC1(a). The 60 product protein was aseptically harvested from the 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 65 L of harvest filtrate was then loaded onto a 400 mL column containing Protein A SEPHAROSE™ resin (Amersham

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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 addition of Tris base and frozen at  $-20^{\circ}$  C.

Several frozen lots of Flt1D2.Flk1D3.FcΔC1(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 SUPERDEX<sup>TM</sup> 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 Flt1D2.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.FcΔC1(a) were used to harvest and purify Flt1D2.VEGFR3D3.FcΔC1(a).

#### Example 23

## Phosphorylation Assay for Transiently Expressed VEGFR2

Primary human umbilical vein endothelial cells (HU-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, Flk-1 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-Flt1D2Flk1D3.FcΔC1(a) 3)-Fc (A40).Flt1D2VEGFR3D3.FcΔC1(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-25 and 26A-26B show the results of this experiment. FIG. 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 55 levels depending on which modified Flt1 receptor is used during the preincubations with VEGF. As is seen in FIG. 25A, at a 1.5 molar excess of either Flt1(1-3)=Fc, Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3.FcΔC1(a) there is complete blockage of receptor stimulation by these three modified Flt receptors as compared to control media challenge. In contrast, transient Flt1D2VEGFR3D3.FcΔC1(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 how be shown to be partially blocking VEGF165-induced stimulation of cell-surface receptors.

In FIG. 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 4 fold molar excess (FIG. 26B) 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.

### 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 fused to the TrkB intracellular kinase domain, thus producing a chimeric molecule. The reason the TrkB intracellular kinase 25 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 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 (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  $37^{\circ}$  C. The following modified Flt receptors Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a), plus an irrelevant receptor termed Tie2-Fc as a negative control, were titrated from 40 40 nM to 20 pM 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 plates were incubated for 72 hrs. at 37° C. end then MTS (Owen's reagent, Promega) added and the plates were incubated for an 45 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 receptor Tie2-Fc does not block VEGF165-induced cell proliferation at any concentration whereas Flt1D2.Flk1D3.FcΔC1(a) block's 1.56 nM 50 VEGF165 with a half maximal dose of 0.8 nM. Flt1(1-3)-Fc and Flt1D2.VEGFR3D3.FcΔC1(a) are less effective in blocking VEGF165 in this assay with the 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

BIACORETM Analysis. The stoichiometry of Flt1D2Flk1D3.Fc $\Delta$ C1(a) and VEGFR1R2-Fc $\Delta$ C1(a) interaction with human VEGF165 was determined by measuring either the level of VEGF saturation binding to the 65 Flt1D2Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a) surfaces or measuring concentration of VEGF165 needed to com-

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pletely prevent binding of Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) to VEGF BIACORE<sup>TM</sup> chip surface.

Modified Flt receptors Flt1D2Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a), were capture with an anti-Fc specific antibody that was first immobilized on a BIACORE<sup>TM</sup> 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.FcΔC1(a) and VEGFR1R2-FcΔC1(a) surfaces at 10 μl/min for one hour. A real-time binding signal was recorded and saturation binding was achieved at end of each infection. Binding stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(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.FcΔC1(a) or VEGFR1R2-FcΔC1(a) molecule (FIG. 28).

In solution, Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) at a concentration of 1 nM (estimated to be 1000 times higher than the KD of the Flt1D2Flk-1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a)/VEGF-165 interaction) were-mixed with varied concentrations of VEGF165. After one hour incubation, concentrations of the free Flt1D2Flk1D3.FcΔC1(a) in solution were measured as a binding signal to an aminecoupled VEGF165 surface. A calibration curve was used to convert the Flt1D2Flk1D3.FcΔC1(a) BIACORE™ binding signal to its molar concentration. The data showed that the addition of 1 nMVEGF165 into the Flt1D2Flk1D3.FcΔC1(a) solution completely blocked Flt1D2Flk1D3.FcΔC1(a) binding to the VEGF165 surface. This result suggested the binding stoichiometry of one VEGF165 molecule per one Flt1D2Flk1D3.FcΔC1(a) molecule (FIG. 29 and FIG. 30). When the concentration of Flt1D2Flk1D3.FcΔC1(a) was plotted as a function of added concentration of VEGF165, the slope of the linear portion was -1.06 for Flt1D2Flk1D3.FcΔC1(a) and -1.07 for VEGFR1R2-FcΔC1 (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.FcΔC1(a) or VEGFR1R2-

Size Exclusion Chromatography. Flt1D2Flk1D3.FcΔC1 (a) was mixed with a 3-fold excess of VEGF165 and the receptor-ligand complex was purified using a Pharmacia SUPEROSE<sup>TM</sup> 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.FcΔC1(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 of Flt1D2Flk1D3.Fc∆C1(a) and VEGF165 were made and peak height or peak integrated intensity was plotted as a function of the concentration of injected protein. The calibration was done under condition identical to one used in separating components of Flt1D2Flk1D3.FcΔC1(a)/VEGF complex. Quantification of the Flt1D2Flk1D3.FcAC1(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.FcΔC1(a) in a complex to be 1:1.

### Example 26

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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 Flt1D2.Flk1D3.FcΔC1(a) (concentration=mg/ml) in molar ratio of 3:1 (VEGF165: 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 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 µl/min. at room temperature. The results of this SEC are shown in FIG. 31. Peak #1 represents the complex and 10 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 dissociative 15 conditions. To separate the components of the receptor-ligand complex and to determine their molar ratio, 50 µl of dissociated complex as, described supra was loaded onto a SUPER-OSETM 12 PC 3.2/30 equilibrated in 6 M GuHCl and eluted temperature. The results of this SEC are shown in FIG. 32. Peak #1 represents Flt1D2Flk1D3.FcΔC1(a) and peak #2 represents VEGF165.

Calculation of Flt1D2Flk1D3.Fc∆C1(a) VEGF165 Complex Stoichiometry. The stoichiometry of the receptor-ligand 25 complex was determined from the peak area or the peak height of the components. Concentrations of VEGF165 and Flt1D2Flk1D3.FcΔC1(a), corresponding to the peak height or peak area, respectively, were obtained from the standard curves for VEGF165, and Flt1D2Flk1D3.FcΔC1(a). To 30 obtain a standard curve, four different concentrations (0.04 mg/ml-0.3 mg/ml) of either component were injected onto a Pharmacia 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 35 curve was obtained by plotting peak area or peak height vs protein concentration. The molar ratio of VEGF165: Flt1D2Flk1D3.FcΔC1(a) determined from the peak area of the components was 1.16. The molar ratio of VEGF165: Flt1D2Flk1D3.FcΔC1(a) determined from the peak height of 40 the components was 1.10.

### Example 27

Determination of the Stoichiometry of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 Complex by Size Exclusion Chromatography with On-Line Light Scattering

Complex preparation. VEGF165 was mixed with CHO 50 by underline in the FIG. 36. transiently expressed Flt1D2.Flk1D3.FcΔC1(a) protein in molar ratio of 3:1 (VEGF165:Flt1D2Flk1D3.FcΔC1(a)) and incubated overnight at 4° C.

Size Exclusion Chromatography (SEC) with On-Line Light Scattering. Size exclusion chromatography column 55 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<sup>TM</sup> 12 HR 10/30 60 column (Pharmacia) equilibrated in PBS buffer and eluted with the dame 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 calcu- 65 lated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor34

ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.FcΔC1(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.FcΔC1(a)/VEGF complex is 1:1 as its corresponds to the sum of molecular weights for Flt1D2Flk1D3.FcΔC1(a) and VEGF165. Importantly, this method conclusively proved that the Flt1D2Flk1D3.FcΔC1 (a)/VEGF165 complex was indeed composed of only one molecule of VEGF165 ligand and only one molecule of the Flt1D2Flk1D3.FcΔC1(a).

### Example 28

### Peptide Mapping of Flt1D2.Flk163.FcΔC1(a)

The disulfide structures and glycosylation sites in with; the same solution at a flow rate 40 μl/min at room 20 Flt1D2.Flk1D3.FcΔC1(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 Flt1D2.Flk1D3.Fc $\Delta$ 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.FcΔC1(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

### Example 29

### Pharmacokinetic Analysis of Modified Flt Receptors

Pharmacokinetic analysis of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a). Balb/c mice (25-30 g) were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.FcΔt1(a), CHO stably expressed Flt1D2.Flk1D3.FcΔC1(a), and CHO transiently expressed 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 detect

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  $T_{max}$  for the transient and stable Flt1D2.Flk1D3.FcΔC1(a) and the transient VEGFR1R2-FcΔC1(a) was 24 hrs. The  $C_{max}$  for Flt1(1-3)-Fc (A40) was 8 μg/ml. For both transients (Flt1D2.Flk1D3.FcΔC1(a) and VEGER1R2-FcΔC1(a)) the  $C_{max}$  was 18 μg/ml and the  $C_{max}$  for the stable VEGFR1R2-FcΔC1(a) was 30 μg/ml.

Pharmacokinetic analysis of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) 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 Flt1D2.Flk1D3.FcΔC1(a) and CHO transiently expressed 15 Flt1D2.VEGFR3D3.FcΔC1(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). The ELISA involves coating an ELISA plate with VEGF  $\,^{20}$ 165, binding the Flt1(1-3)-Fc; Flt1D2.Flk1D3.FcΔC1(a) or Flt1D2.VEGFR3D3.FcΔC1(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 whereas. Flt1D2.Flk1D3.FcΔC1(a) Flt1D2.VEGFR3D3.FcΔC1(a) were detectable for 15 days of 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.FcΔC1(a) to 35 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 fibrosarcoma cell line (ATCC accession no. CCL-121) and the rat C6 40 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 Flt1D2.Flk1D3.FcΔC1(a) (at 25 mg/Kg or as indicated in FIGS. 39 and 40) was, given on the clay of tumor implanta- 45 tion. 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  $(2\times/wk)$ for a period of 2 weeks. After 2 weeks, animals were perfused with fixative, tumors were removed and samples were 50 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.FcΔC1(a) significantly reduced the growth of tumors formed by HT-1080 and C6 cells. The results of these experiments are shown in FIG. 55 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

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regression. Indeed, in situ hybridization studies in the reproductive tissues provided the first clear evidence that VEGF acts as a mediator of physiological engiogenesis 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 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 al., 1995; Shifren et al., 1996; Guidi et at, 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., 1996). 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 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 station, 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., 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 60 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 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.

Assessment of VEGF-Induced Uterine Hyperpermeability. Pregnant mare's serum gonadotrophin (PMSG) was 5 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 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 Flt1D2.VEGFR3D3.FcΔC1(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) Flt1D2.VEGFR3D3.FcΔC1(a) at 25 mg/kg at 1 hr after PMSG injection results in about a 50% inhibition of the 20 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 progesterone as a readout. Pregnant mare's serum gonadotrophin (PMSG) is injected subcutaneously (5 IU) to induce ovulation in prepubertal females rats. This results in a fully functioning corpus luteum containing a dense, network of blood 30 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 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 40 Flt1D2.Flk1D3.FcΔC1(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 FIG. 42A-42B.

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### 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  $T_m$  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 pM to 0.1 pM. 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-FcΔC1(a), Flt1-(1- $3_{NAS}$ )-Fc, Flt1(1-3<sub>R→C</sub>)-Fc and Tie2-Fc. Flt1(1-3<sub>NAS</sub>)-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- $3_{R \to C}$ )-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.FcΔC1(a), followed by Flt1(1-3)-Fc and Flt1(1-3)-Fc (A40) and then by Flt1(1- $3_{R\to C}$ )-Fc, Flt1(1-3 $_{NAS}$ )-Fc and Flt1D2VEFGFR3D3-Fc $\Delta$ C1 (a). Tie2Fc has no affinity for VEGF165.

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                                                                       96
Cys Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro
             20
                                25
gaa ctg agt tta aaa ggc acc cag cac atc atg caa gca ggc cag aca
                                                                      144
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	gtt Val															1008		
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	Val			325				-	330	-				335	
	ГÀв		340	_	-			345	-			-	350		
	Leu	355					360					365	-		
_	Thr 370					375					380	-			
385	Val				390					395					400
-	Val			405			•		410					415	-
			420					425					430		Asp
	Leu	435	-	-		-	440	-	-			445	-		
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Thr	Thr	Pro	Pro	Val	Leu	Asp	Ser	Asp	Gly	Ser	Phe	Phe	Leu	Tyr	Ser

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gaa atg Glu Met 65															240	
tgt gga Cys Gly															288	
gct caa Ala Glr															336	
cct act Pro Thr															384	
agt gat Ser Asp 130	Thr														432	
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acg tca Thr Ser				Thr											528	
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atc ata Ile Ile		Asn													624	
gca aca Ala Thr 210	Val					Tyr									672	
caa acc Gln Thr															720	

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Lys	Ala	Gly	Pro	Gly 325	Glu	Pro	Lys	Ser	330 330	Asp	Lys	Thr	His	Thr 335	Сув
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	aac Asn															384	
	agc Ser			_		_				_					-	432	

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togg agt tac cet gat gas att gac can age and tee cat gee and tan 11							55										J <b>T</b>
ted aat tigt act get acc act coc tig asc acg aga git can atg acc too lead Ann Cys Thr Als Thr Thr Pro Lew Ann Thr Ars Val Clin Met Thr 180 1150 1150 1150 1150 1150 1150 1150												-	con	tin <sup>.</sup>	ued		
Lead Amo Cye Thr Ala Thr Thr Pro Leu Am Thr Arg Val Gln Met Thr  165  169 agt tac cott gat gaa att gac caa age aat too cat got asc atta  169 agt tac cott gat gaa att gac caa age aat too cat got asc atta  160 cln Ile Amp Gln Ser Amn Ser Him Ala Amn Ile  160 cln Ile Amp Gln Ser Amn Ser Him Ala Amn Ile  160 cln Ile Amp Gln Ser Amn Ser Him Ala Amn Ile  160 cln Ile Amp Gln Ser Amn Ser Him Ala Amn Ile  160 cln Ile Amp Gln Ser Amn Ser Him Ala Amn Ile  160 cln Ile Amp Gln Ser Amn Ser Him Ala Amn Ile  160 cln Ile Amp Gln Ser Amn Ser Him Ala Amn Ile  160 cln Ile Amp Gln Ser Amn Ser Him Ala Amn Ile  160 cln Ile Amp Gln Ser Amn Ile Amn Ile Amn Ile  160 cln Ile Amp Gln Ser Amn Ile Amn Ile Amn Ile  160 cln Ile Amp Gln Ser Amn Ile Amn Ile Amn Ile  160 cln Ile Amp Gln Ser Phe Iley Ser Val Amn  160 cln Ile Amn Ile Amp Gln Ser Phe Iley Ser Val Amn  160 cln Ile Amn Ile Amp Gln Ser Phe Iley Ser Val Amn  160 cln Ile Amn Ile Amp Gln Ser Phe Iley Ser Val Amn  160 cln Ile Amp Gln Ser Phe Iley Ser Val Amn  160 cln Ile Amn Ile Amp Gln Ser Phe Iley Ser Val Amn  160 cln Ile Amn Ile Amp Gln Ser Phe Iley Ser Val Amn  160 cln Ile Amn Ile Amp Gln Ser Phe Iley Ser Val Amn  160 cln Ile Amn Ile Amp Gln Ser Phe Iley Ser Val Amn  160 cln Ile Amp Gln Ser Phe Iley Ser Val Amn  160 cln Ile Amn Ile Amp Gln Ser Phe Iley Ser Val Amn  160 cln Ile Amn Ile Amp Gln Ser Phe Iley Ser Val Amn  160 cln Ile Amn Ile Amp Gln Ser Phe Iley Ser Val Amn  160 cln Ile Amn Ile Amp Gln Ser Phe Iley Ser Ile Ile Ile Ile  160 cln Ile Amn Ile Amp Gln Ser Phe Iley Ser Ile Ile  160 cln Ile Amn Ile Ile Amp Gln Ser Phe Iley Ser Ile Ile  160 cln Ile Amn Ile Ile Thr Ile Thr Cyp Val Amn Ile Ile Ile Ile  160 cln Ile Amn Ile Ile Thr Ile Ile Amp Gln Ser Ile  160 cln Ile Amn Ile Ile Thr Val Ile Ile Ill Ile Ill Ile Ill Ile Ill Ill		130					135					140					
Try Pro Amp Giu Ile Amp Clu Ser Amn Ser Him Ala Amn Ile 165 170 165 176 176 176 176 176 176 176 176 176 176						Thr					Thr					Thr	480
the try Ser val Leu Thr ILe Aep Lys Met Glin Asm Lys Aep Lys Gly 180 185 185 186 187 187 188 188 188 188 188 188 188 188					Asp					Ser					Asn		528
Leu Tyr Thr Cye Arg Val Arg Ser Giy Pro Ser Phe Lys Ser Val Aen 195 200 205 206 207 208 208 208 207 208 208 208 208 208 208 208 208 208 208				Val					Lys					Asp			576
The Ser Val His Die Tyr Amp Lyp Ala Giy Pro Giy Giu Pro Lyp Ser 210 225 226 226 226 227 246 225 235 230 225 240 225 240 225 235 230 235 240 226 240 226 226 226 226 226 226 226 226 226 22			Thr					Ser					Lys				624
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Tyr Arg Val Val Ser Val Leu Thr Val Leu Thr Val Leu His Gln Asp Trp Leu Asn 320  ggg aag gag tac aag tgc aag gtc tcc aac aag gcc ctc cca gcc ccc 1008  Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro 335  atc gag aaa acc atc tcc aaa gcc aaa ggg cag ccc cga gaa cca cag 1056  Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln 345  gtg tac acc ctg ccc cca tcc cgg gat gag ctg acc aag aac cag gtc Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val 355  agg ctg acc tgc ctg gtc aaa ggc ttc tat ccc agg gac atc gcc gtg 356  Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val 370  gag tgg gag agg ag agc aat ggg cag ccg gag aac aac tac aag acc acg ct 1200  gag tgg gag agc atc ggg cag ccg gag aac aac tac aag acc acg ct 1200  gag tgg gag agc atc ggc gg ag aac aac tac aag acc acg ct 1200  gag tgg gag agc atc gg gac ccg gag aac aac tac aag acc acg ct 1200  gag tgg gag agc ag agc atc gg gac acc gag aac acc tac aag acc acg ct 1200  gag tgg gac aag agc tcc gac ggc tcc ttc ttc ctc tac agc aag ctc acc acc 1248  Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr 415  gtg gac aag agc agg ctg cac aac cac cac cac ctc ctc ctg acc gac aag aac acc acc gtc Ctc tcc tcc tcc tcc tcc tcc ctg acc ctc ctc ctc ctc ctc ctc ctc ctc ctc		His					Lys					Gln					912
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Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr 405  gtg gac aag agc agg tgg cag cag ggg aac gtc ttc tca tgc tcc gtg  Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val 420  atg cat gag gct ctg cac aac cac tac acg cag aag agc ctc tcc ctg  Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu						Gly					Asn					Pro	1200
Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val 420 425 430 atg cat gag gct ctg cac aac cac tac acg cag aag agc ctc tcc ctg 1344 Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu					Ser					Phe					Leu		
Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu	Val	Āsp	Lys	Ser 420	Arg	Trp	Gln	Gln	Gly 425	Asn	Val	Phe	Ser	Cys 430	Ser	Val	
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Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val

												acr	tin <sup>.</sup>	1104			
												COII	CIII	ueu			
	370					375					380						
Glu 385	Trp	Glu	Ser	Asn	Gly 390	Gln	Pro	Glu	Asn	Asn 395	Tyr	Lys	Thr	Thr	Pro 400		
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							atc Ile									288	
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							caa Gln 120									384	
							aaa Lys									432	
							ccc Pro									480	
							aat Asn									528	
							gcc Ala									576	
							gac Asp 200									624	

59

-continued  -continued  -agg agt ggs cat ta tite ama tet git ama can tae tae get at at tat  -continued						33										00	
Arg Ser Gily Pro Ser Ple Lyw Ser Val Am Th Ser Val His Ile Tyy 210  gat aas gos gos cog gos gos cos aas tot tot gos aas act cac aca able also also gos cog gos gos cos aas tot tot gos aas act cac aca by Pro Cly Pro Cly Pro Cly Ser Cyc App Lyw Thr His Thr 225  tot coc cog tog cos aga cot gas cot cat gos gos gos cog tos got tot cyc coc cog tog cos aas coc aag gos cot cat gos gos gos cot cat gos act cat cat gos gos acc cot cleu Phe Pro Pro Cly Pro No Lyw Am Thr Leu Het Ile Ser Arg Thr Pro 275  gog got cac tog gos got gos gos gos gos gos coc coc act gos acc cot aga gos cot Leu Phe Pro Pro Lyw Pro Lyw Am Thr Leu Het Ile Ser Arg Thr Pro 276  gog got cac tog gos got gos gos gos gos gos gos coc coc act act gos gos acc coc act coc year year and to cac tog gos gos gos gos gos gos gos gos gos g											-	con	tin	ued			
Amp Lyo Ala dily Pro Gity Sin Pro Lyo Ser Cyo Amp Lyo Thr His Thr 225 226 226 226 226 226 226 226 226 226	Arg Ser	Gly				Lys					Ser					672	
Cyp Pro Pro Cyp Pro Ala Pro Glu Leu Leu Cly Gly Pro Ser Val Phe 245	Asp Lys				Gly					CAa					Thr	720	
Leu Phe Pro Pro Lye Pro Lye Ap 266  gag gtc aca tgc gtg gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc Glu Val Thr Cye Val Val Val Aap Val Ser His Glu App Pro Glu Val 270  aag ttc aac tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca Lye Phe Ann Trp Tyr Val App Gly Val Glu Val His Ann Ala Lye Thr 280  aag ctc aac tgg tac gac gac aca ga gtg gtg gtg gtg gtg gtg gtg aag cg cgg gag gag cag tac aac aca agc acq tac cgt gtg gtc agc gtc Lye Pro Arg Glu Glu Glu Gln Tyr Ann Ser Thr Tyr Arg Val Val Ser Val 305  aag ctc acc ag gac tgg ctg sta and tggc aag gtg tcc acc Leu Thr Val Leu His Gln App Trp Leu Ann Gly Lye Glu Tyr Lye Cye 325  aag gtc tcc acc aag gac tgg ctc cac acc ggc acc acc atc gag aac acc acc tcc Lye Val Ser Ann Lye Ala Leu Pro Ala Pro Ile Glu Lye Thr Ile Ser 340  aaa gcc aaa gag cag ccc ga gaa cca cag gtg tac acc ctg ccc cca Lye Wal Ser Ann Lye Ala Leu Pro Gln Val Tyr Thr Leu Pro Pro 345  aaa gcc aaa gag cag acc cag gac cca gag dta acc acc tgc ccc cca Lye Gly Gln Pro Arg Glu Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro 355  aaa ggc ttc tat ccc acc aga gac cag aga cca gag dta acc acc tgc ctg ccc cca Lye Wal Sar App Glu Leu Thr Lye Ann Gln Val Ser Leu Thr Cye Leu Val 370  370  380  aac gcc gag stag ctg acc aca aca aca cag ctg acc tg ctg ctg ccc cca Lye Wal Ala Lye Ann Lye Ann Glu Tyr Thr Leu Pro Pro 365  aac gcc gag gag acc acc acg acc acc acg ctg acc tgc ctg gtc 1152  aac gc ctc tat ccc acc acc acc acc acc gtg gag dac acc acc gcc Cag cag cag gag acc acc acc acc acc gcc g				Pro					Leu					Val		768	
Singurary   Sing			Pro					Thr					Arg			816	
Lys Phe Ann Trp Tyr Val Asp Gly Val Glu Val His Ann Ala Lys Thr 290  aag ccg cgg gag gag cag tac aac agc acg tac cgt gtg gtc agc gtc Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val 305  all Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val 310  ctc acc gtc ctg cac cag gac tgg ctg at gg ctg at gg tag at gag tac agg gtc Leu Thr Val Leu His Gln Asp Trp Leu Ann Gly Lys Glu Tyr Lys Cyc 325  aag gtc tec aac aaa gec ctc cca gec ccc atc gag aa aac ac atc tec Lys Val Ser Ann Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser 340  aaa gec aaa ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc ca Lys Val Ser Ann Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser 340  aaa gec aaa ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc ca Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro 350  tcc cgg gat gag ctg acc aag aac cag gtc agc ctg ctg ctg acc tgc ctg Ser Arg Ann Glu Leu Thr Lys Ann Gln Val Ser Leu Thr Cys Leu Val 370  aaa ggc ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat ggg 1200  Lys Gly Phe Tyr Pro Ser Ann Ile Ala Val Glu Trp Glu Ser Ann Gly 395  aag gcc gg gag aac aac tac aag acc acg ct ccc gtg ctg gac tcg gac Cln Pro Glu Ann Ann Tyr Lys Thr Thr Pro Pro Val Leu Ann Ser Ann 400  cag ccg gag aac aac tac aag acc acg ct ccc gtg gag agc agc agc Cln Pro Glu Ann Ann Tyr Lys Thr Thr Yar Pro Pro Val Leu Ann Ser Ann 400  cag cag gag gaa gac gtc ttc tca tag cag ctc acc gtg gac agc agc agc agc Cln Pro Glu Ann Ann Tyr Lys Er Lys Leu Thr Val Ann Lys Ser Arg Trp 420  cag cag gag gaa gac gtc ttc tca tag tag ctc gtg gag gag agc agc agc agc Cln Gln Gly Ann Val Phe Ser Cys Ser Val Met His Glu Ala Leu His 435  440  cac cac tac acg cag aa agc ctc tcc ctg tct ccg ggt aaa Ann His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys 450  c210> SEQ ID NO 8  c211> LEUNGTH: 462  c212> TTPE: PRT  c213> ORGANISN: Homo sapiens  c400> SEQUENCE: 8  Met Val Ser Tyr Ty Ann Thr Gly Val Leu Leu Cys Ala Leu Leu Ser 10  Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu		Thr					Asp					Asp				864	
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Leu Thr Val Leu His Gln App Trp Leu Ann Gly Lye Glu Tyr Lye Cye 325 330  aag gto toc aac aaa goc cto coa goc ccc atc gag aaa acc atc toc Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser 340  aaa gog caa ag ggg cag coc cga gaa cca cag gtg tac acc ctg ccc cca Lye Ala Lye Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Pro 355  tcc cgg gat gag ctg acc aag aac aag acc ag gtc agc ctg acc tgc ctg ctg ser Arg App Glu Leu Thr Lye App Gln Val Ser Leu Thr Cys Leu Val 370  aaa ggt tct tat ccc agc gaa atc gcc gtg gag gg gaa gac aat ggg Lye Gly Phe Tyr Pro Ser App Ile Ala Val Glu Trp Glu Ser Asn Gly 385  aaa ggt tct tat ccc agc gaa acc acg ctc cc gtg ctg gaa gac aat ggg Lye Gly Phe Tyr Pro Ser App Ile Ala Val Glu Trp Glu Ser Asn Gly 385  aaa ggt tct tat ccc agc gaa acc acg cct ccc gtg ctg gac toc gcc gag aaa gac aac tac aaa acc acg cct ccc gtg ctg gac toc gcc Gln Pro Glu Aen Aen Tyr Lye Thr Thr Pro Pro Val Leu App Ser Aep 400  agc ccg gag aac ac tac aca gac acc acg ctc ccc gtg ctg gac toc gcc Gln Pro Glu Aen Aen Tyr Lye Thr Thr Pro Pro Val Leu App Ser Aep 410  ggc tcc tct ttc ctc tac acg aag ctc acc gtg gac aaa gag agg agg Lye Gly Ser Phe Phe Leu Tyr Ser Lye Leu Tr Val Aep Lye Ser Arg Trp 420  acag cag ggg aac gtc ttc tca tgc tcc gtg atg gat gag gct ctg cac Gln Gly Aen Val Phe Ser Cya Ser Val Met His Glu Val Leu His 445  aac cac tac acg cag aag agc ctc tcc ctg tct ccg ggt aaa Aen His Tyr Thr Gln Lye Ser Leu Ser Leu Ser Pro Gly Lye 450  460  450  Age  **C210> SEQ ID NO 8  **211> LENGTH: 462 **212> TYPE: PRT 213> ORGANISM: Homo sapiens  **400> SEQUENCE: 8  Met Val Ser Tyr Trp Aep Thr Gly Val Leu Leu Cys Ala Leu Leu Ser 1 5  Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu	Lys Pro				Gln					Tyr					Val	960	
Lye Val Ser Ann Lye Ala Leu Pro Ala Pro 11e Slu Lye Thr 11e Ser 340  aaa ggc aaa ggg cag cac cag aga cac cac				His					Asn					Lys		1008	
Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro 365  Ser Arg Aap Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val 370  aaa ggc ttc tat ccc agc gac atc gcc gtg gac tgg gac ag gg gag tgg gag ag gac atc gcc gtg gac tgg gac tgg gag tgg gag ag aca atc gcc gtg gac tgg gac aca tgg gad tgg gag ag gac atc gcc gtg gac tgg gac aca tgg gad tgg gag aca atc gcc gtg gac tgg gag tgg gag tgg gag tgg gac aca gcc gcg gag aca acc gcc gtg gac tgg gac tgc gac acc gcg gag aca acc gcc gtg gac tgc gac acc gcg gac aca acc gcc gtg gac tgc gac tcc gac gla gac tac gag gac aca acc tac aag acc acc gtg gac tgc gac tcc gac gla gac tac gac gcd gac tcc gac gla gac tac gac gac acc gcg gag aca aca acc tac acc gtg gac acc gcg gac tcc gac gla gac tac gac gac gac gac gac gac acc gcg gac acc gcc g			Asn					Ala					Thr			1056	
Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val 370       1200       1200         aaa ggc ttc tat ccc agc gac act geg gag tag gan act gag gag tag gan act gag gag tag gan act gag gag tag gag g		Lys					Glu					Thr				1104	
Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly 395  cag ccg gag aac aac aac tac aag acc acg cct ccc gtg ctg gac tcc gac Clu Asp Asp Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp 415  ggc tcc ttc ttc ctc tac agc aag ctc acc gtg gac aag agc agg tgg Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp 420  cag cag ggg aac gtc ttc tc at tac at ge cc gtg gac aag agc ctc acc gtg gac aag agc agg tgg Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp 430  cag cag ggg aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac Gln Gln Gly Asp Val Phe Ser Cys Ser Val Met His Glu Ala Leu His 445  aac cac tac acg cag aag agc ctc tcc ctg tct ccg ggt aaa Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys 455  tga 1386 <pre> </pre> 1386 <pre> <pre> <pre></pre></pre></pre>	Ser Arg	Asp				Lys					Leu					1152	
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Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp 420  cag cag ggg aac gtc ttc tca tgc tcc gtg atg cat gal gcc ctg cac Gln Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His 435  aac cac tac acg cag aag agc ctc tcc ctg tct ccg ggt aaa Asn His Tyr Thr Gln Lys Ser Leu Ser Pro Gly Lys 450  tga  1386  210				Asn					Pro					Ser		1248	
Gln Gln Gln Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His 445  aac cac tac acg cag aag agc ctc tcc ctg tct ccg ggt aaa 1386  Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys 450  tga 1389 <pre> &lt;210 &gt; SEQ ID NO 8 &lt;2211 &gt; LENGTH: 462 &lt;2212 &gt; TYPE: PRT &lt;213 &gt; ORGANISM: Homo sapiens </pre> <400 > SEQUENCE: 8  Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser 15  Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu  Tyn Trp Asp Thr Gly Ser Ser Gly Gly Arg Pro Phe Val Glu  Tyn Trp Asp Trp Asp Thr Gly Ser Ser Gly Gly Arg Pro Phe Val Glu  Tyn Trp Asp Trp Asp Trp Asp Trp Arg Pro Phe Val Glu  Tyn Trp Asp Trp Asp Trp Arg Pro Phe Val Glu  Tyn Trp Asp Trp Asp Trp Arg Pro Phe Val Glu  Tyn Trp Asp Trp Asp Trp Arg Pro Phe Val Glu  Tyn Trp Asp Trp Arg Pro Phe Val Glu  Tyn Trp Arg Pro Phe Va			Phe					Leu					Ser			1296	
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		Ser	Tyr		Asp	Thr	Gly	Val		Leu	Cys	Ala	Leu		Ser		
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												COII	CIII	aca	
Met	Tyr	Ser 35	Glu	Ile	Pro	Glu	Ile 40	Ile	His	Met	Thr	Glu 45	Gly	Arg	Glu
Leu	Val 50	Ile	Pro	Cys	Arg	Val 55	Thr	Ser	Pro	Asn	Ile 60	Thr	Val	Thr	Leu
Eys	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
Ile	Gly	Leu	Leu 100	Thr	Cys	Glu	Ala	Thr 105	Val	Asn	Gly	His	Leu 110	Tyr	Lys
Thr	Asn	Tyr 115	Leu	Thr	His	Arg	Gln 120	Thr	Asn	Thr	Ile	Ile 125	Asp	Val	Gln
Ile	Ser 130	Thr	Pro	Arg	Pro	Val 135	Lys	Leu	Leu	Arg	Gly 140	His	Thr	Leu	Val
Leu 145	Asn	Cys	Thr	Ala	Thr 150	Thr	Pro	Leu	Asn	Thr 155	Arg	Val	Gln	Met	Thr 160
Trp	Ser	Tyr	Pro	Asp 165	Glu	Lys	Asn	Lys	Arg 170	Ala	Ser	Val	Arg	Arg 175	Arg
Ile	Asp	Gln	Ser 180	Asn	Ser	His	Ala	Asn 185	Ile	Phe	Tyr	Ser	Val 190	Leu	Thr
Ile	Asp	Lуs 195	Met	Gln	Asn	Lys	Asp 200	Lys	Gly	Leu	Tyr	Thr 205	Cys	Arg	Val
Arg	Ser 210	Gly	Pro	Ser	Phe	Lys 215	Ser	Val	Asn	Thr	Ser 220	Val	His	Ile	Tyr
Asp 225	Lys	Ala	Gly	Pro	Gly 230	Glu	Pro	Lys	Ser	Сув 235	Asp	Lys	Thr	His	Thr 240
CAa	Pro	Pro	Cys	Pro 245	Ala	Pro	Glu	Leu	Leu 250	Gly	Gly	Pro	Ser	Val 255	Phe
Leu	Phe	Pro	Pro 260	ГÀв	Pro	ГАв	Asp	Thr 265	Leu	Met	Ile	Ser	Arg 270	Thr	Pro
Glu	Val	Thr 275	Cha	Val	Val	Val	Asp 280	Val	Ser	His	Glu	Asp 285	Pro	Glu	Val
rAa	Phe 290	Asn	Trp	Tyr	Val	Asp 295	Gly	Val	Glu	Val	His 300	Asn	Ala	ГÀа	Thr
305	Pro	Arg	Glu	Glu	Gln 310	Tyr	Asn	Ser	Thr	Tyr 315	Arg	Val	Val	Ser	Val 320
Leu	Thr	Val	Leu	His 325	Gln	Asp	Trp	Leu	Asn 330	Gly	ГÀв	Glu	Tyr	335	Cys
Lys	Val	Ser	Asn 340	Lys	Ala	Leu	Pro	Ala 345	Pro	Ile	Glu	Lys	Thr 350	Ile	Ser
rAa	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	CÀa	Leu	Val
185 385	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	Lys	Thr	Thr	Pro 410	Pro	Val	Leu	Asp	Ser 415	Asp
Gly	Ser	Phe	Phe 420	Leu	Tyr	Ser	Lys	Leu 425	Thr	Val	Asp	Lys	Ser 430	Arg	Trp
Gln	Gln	Gly 435	Asn	Val	Phe	Ser	Cys 440	Ser	Val	Met	His	Glu 445	Ala	Leu	His
Asn	His 450	Tyr	Thr	Gln	Lys	Ser 455	Leu	Ser	Leu	Ser	Pro 460	Gly	Lys		

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			aaa tta aaa gat Lys Leu Lys Asp 30	
		His Ile Met	caa gca ggc cag Gln Ala Gly Gln 45	
			aaa tgg tct ttg Lys Trp Ser Leu 60	
			ata act aaa tct Ile Thr Lys Ser	
			tta acc ttg aac Leu Thr Leu Asn 95	
			aaa tat cta gct Lys Tyr Leu Ala 110	
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		. Glu Met Tyr	agt gaa atc ccc Ser Glu Ile Pro 140	
			att ccc tgc cgg Ile Pro Cys Arg	
			ttt cca ctt gac Phe Pro Leu Asp 175	
			agt aga aag ggc Ser Arg Lys Gly 190	
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		Lys Thr Asn	tat ctc aca cat Tyr Leu Thr His 220	
			aca cca cgc cca Thr Pro Arg Pro	
			tgt act gct acc Cys Thr Ala Thr 255	
			tac cct gat gaa Tyr Pro Asp Glu 270	

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			-conti	nued	
	n Ala Ser Val	agg cga cga att Arg Arg Arg Ile 280			
		gtt ctt act att Val Leu Thr Ile 295			
		tgt cgt gta agg Cys Arg Val Arg			
		cat ata tat gat His Ile Tyr Asp 330			
		act cac aca tgc Thr His Thr Cys 345		o Ala Pro	
	ı Gly Gly Pro	tca gtc ttc ctc Ser Val Phe Leu 360			
		cgg acc cct gag Arg Thr Pro Glu 375			
		cct gag gtc aag Pro Glu Val Lys			
		gcc aag aca aag Ala Lys Thr Lys 410			
		gtc agc gtc ctc Val Ser Val Leu 425		s Gln Asp	
	n Gly Lys Glu	tac aag tgc aag Tyr Lys Cys Lys 440			
		acc atc tcc aaa Thr Ile Ser Lys 455			
		ctg ccc cca tcc Leu Pro Pro Ser			
		tgc ctg gtc aaa Cys Leu Val Lys 490			
atc gcc gtg Ile Ala Val	g gag tgg gag L Glu Trp Glu 500	agc aat ggg cag Ser Asn Gly Gln 505	ccg gag aac aa Pro Glu Asn As 51	n Tyr Lys	
	Pro Val Leu	gac tcc gac ggc Asp Ser Asp Gly 520			
		agc agg tgg cag Ser Arg Trp Gln 535			
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010 070 7					

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67

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					0)										
										-	con	tin	ued		
			405					410					415		
Asn Ser 1	Chr	Tyr	Arq	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	
		420	5				425					430			
Trp Leu A	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn	Lys	Ala	Leu	
4	135					440					445				
Pro Ala E	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly	Gln	Pro	Arg	
450					455					460					
Glu Pro (	3ln	Val	Tyr		Leu	Pro	Pro	Ser	_	Asp	Glu	Leu	Thr	-	
465				470					475					480	
Asn Gln V	/al	Ser		Thr	Cys	Leu	Val		Gly	Phe	Tyr	Pro		Asp	
			485					490					495		
Ile Ala V		Glu 500	Trp	Glu	Ser	Asn	Gly 505	Gln	Pro	Glu	Asn	Asn 510	Tyr	rAa	
		500					505					310			
Thr Thr E	Pro 515	Pro	Val	Leu	Asp	Ser 520	Asp	Gly	Ser	Phe	Phe 525	Leu	Tyr	Ser	
Lys Leu 1 530	Chr	Val	Asp	ГÀв	Ser 535	Arg	Trp	Gln	Gln	Gly 540	Asn	Val	Phe	Ser	
													_		
Cys Ser V 545	/al	Met	His	Glu 550	Ala	Leu	His	Asn	His 555	Tyr	Thr	Gln	Lys	Ser 560	
		a -	ъ.		Ŧ.										
eu Ser I	₋eu	ser	Pro 565	GIY	гув										
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agettggg	ge t	geag	gree	ga t	egae	teta	g agg	gate	gate	ccc	gggc	gag	etega	аассе	g 60
aaccacc														g ctg a Leu	110
	1	v a.	. 561	L IY.	5	6 vel	, 111	L GI	y va.	1		u Cyi	5 A10	и пец	
tc agc t	at	ata	att	ata	202	aa s	tat	act	taa	aas	aat	202	cct	tta	158
eu Ser (															130
15				20					25					30	
gta gag a															206
/al Glu N			Ser					Ile					Glu		
			35					40					45		
agg gag d															254
Arg Glu I	∟eu	Val 50	11e	Pro	cys	Arg	Val 55	Thr	ser	Pro	Asn	Ile 60	Tnr	val	
				_				_,	_,						2.2.
act tta a Thr Leu I															302
	65	•				70					75	•	•	J	
ata atc t	aa	gac	aqt	aga	aaq	aac	ttc	atc	ata	tca	aat	qca	acq	tac	350
Ile Ile 1					Lys					Ser					
80					85					90					
aaa gaa a				_		_	_	-		-				_	398
Lys Glu 1 95	[le	Gly	Leu	Leu 100	Thr	Cys	Glu	Ala	Thr 105	Val	Asn	Gly	His	Leu 110	
tat aag a Tyr Lys 1															446
			115				9	120					125	Р	
gtg gtt d	ata	ag†	ככמ	tet	cat	aus	att	gaa	ct a	tet	att	aas	ass	aan	494
yal Val I															323
		130					135					140			

71 72 -continued ctt gtc tta aat tgt aca gca aga act gaa cta aat gtg ggg att gac Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile Asp 145 150 155ttc aac tgg gaa tac cct tct tcg aag cat cag cat aag aaa ctt gta Phe Asn Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu Val 165 aac cga gac cta aaa acc cag tct ggg agt gag atg aag aaa ttt ttg Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe Leu 180 185 age ace tta act ata gat ggt gta ace egg agt gae caa gga ttg tae Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu Tyr 686 200 acc tgt gca gca tcc agt ggg ctg atg acc aag aag aac agc aca ttt Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr Phe gtc agg gtc cat gaa aag ggc ccg ggc gac aaa act cac aca tgc cca Val Arg Val His Glu Lys Gly Pro Gly Asp Lys Thr His Thr Cys Pro 225 230 235 782 ccg tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe 245 ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag gtc Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val 265 aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe  $\,$ 926 280 aac tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro 974 1022 cgg gag gag cag tac aac agc acg tac cgt gtg gtc agc gtc ctc acc Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr 310 gtc ctg cac cag gac tgg ctg aat ggc aag gag tac aag tgc aag gtc 1070 Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val 325 tcc aac aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc aaa gcc Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala 340 aaa ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc cgg Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg 1166 gat gag ctg acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa ggc Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat ggg cag ccg Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro gag aac aac tac aag acc acg cct ccc gtg ctg gac tcc gac ggc tcc Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser 405 ttc ttc ctc tat agc aag ctc acc gtg gac aag agc agg tgg cag cag Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln ggg aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His 1406

tac acg cag aag agc ctc tcc ctg tct ccg ggt aaa tgagcggccg

455

Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys

450

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74

73

c 1453

<210> SEQ ID NO 12

<211> LENGTH: 458 <212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 12

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Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu 50 60

Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile 65  $\phantom{\bigg|}70\phantom{\bigg|}70\phantom{\bigg|}75\phantom{\bigg|}75\phantom{\bigg|}75$ 

Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu  $85 \hspace{0.5cm} 90 \hspace{0.5cm} 95$ 

Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Val 115 \$120\$

Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu Lys Leu Val 130 135 140

Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile Asp Phe Asn 145  $\phantom{\bigg|}$  150  $\phantom{\bigg|}$  150  $\phantom{\bigg|}$  155  $\phantom{\bigg|}$  160

Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu Val Asn Arg

Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe Leu Ser Thr 180  $$185\$ 

Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu Tyr Thr Cys 195  $\phantom{\bigg|}200\phantom{\bigg|}$ 

Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr Phe Val Arg 210 215 220

Val His Glu Lys Gly Pro Gly Asp Lys Thr His Thr Cys Pro Pro Cys 225 \$230\$

Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro 245  $\phantom{-}250\phantom{0}$  255

Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys  $260 \hspace{1.5cm} 265 \hspace{1.5cm} 265 \hspace{1.5cm} 270 \hspace{1.5cm}$ 

Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp 275 280 285

Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu 305 \$310 \$310 \$315

His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn

Ive Ala Leu Pro Ala Pro Ile Clu Ive Thr Ile Ser Ive Ala Ive Clu

Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly  $340 \hspace{1.5cm} 345 \hspace{1.5cm} 350 \hspace{1.5cm}$ 

Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu 355 360 365

						_											
											_	con	tin	ıed			
Leu	Thr 370	Lys	Asn	Gln	Val	Ser 375	Leu	Thr	CAa	Leu	Val 380	rys	Gly	Phe	Tyr		
Pro 385	Ser	Asp	Ile	Ala	Val 390	Glu	Trp	Glu	Ser	Asn 395	Gly	Gln	Pro	Glu	Asn 400		
Asn	Tyr	Lys	Thr	Thr 405	Pro	Pro	Val	Leu	Asp 410	Ser	Asp	Gly	Ser	Phe 415	Phe		
Leu	Tyr	Ser	Lys 420	Leu	Thr	Val	Asp	Lys 425	Ser	Arg	Trp	Gln	Gln 430	Gly	Asn		
Val	Phe	Ser 435	Cys	Ser	Val	Met	His 440	Glu	Ala	Leu	His	Asn 445	His	Tyr	Thr		
Gln	Lys 450	Ser	Leu	Ser	Leu	Ser 455	Pro	Gly	Lys								
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							gga Gly									158	
							ccc Pro									206	
							cgg Arg									254	
							gac Asp 70									302	
							ggc Gly									350	
							tgt Cys									398	
							cat His									446	
							tcg Ser									494	
	Val						tgg Trp 150									542	
							aag Lys									590	
							acc Thr									638	

686

acc atc cac aac gtc agc cag cac gac ctg ggc tcg tat gtg tgc aag

The file His Aen Val Ser Gln His Aep Leu Gly Ser Try Val Cye Lye 205 goc aac aac ggc atc cag cgs tit egg geg agc acc gag gic att gig geg ge acc acc gag gic att gig geg acc acc gag gic att gig ged acc acc gag gic att gig 734 la Aen Aen Mil y lie Gln Arey Phe Arg Glu Ser Try Glu Val 11e Val 200 cat gaa att ggc cog ggc gac aa act cac aca toc cac cog toc ca 782 Rile Gla Aen Gly Pro Gly Aep Lye The His Try Cye Pro Pro Cye Pro 225 gas cot gas ctc ctg ggg ggs cog toc gic tit ctc tot coc cc aaa aa 830 ala Pro Glu Leu Cly Gly Gly Pro Ser Val Phe Leu Phe Pro Dro Lye 243 ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Dro Lye 243 ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Dro Lye 243 ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Dro Lye 243 ala Pro Glu Val Try Leu Phe. Ile Ser Arg Thr Pro Glu Val Try 670 ala Pro Lye App Thr Leu Phe. Ile Ser Arg Thr Pro Glu Val Try 670 ala Seg gic gag gic gag cac act gag gac cac tag gic aca tig gic gag gag gag yal ser gag gac gac gag gag gag acc acc acc gag gac acc ac							, ,											, 0	
goc and and gog at cong gog the cong gog and gog and gog gog gog and and gog gog gog gog gog gog gog gog gog go												-	con	tin <sup>.</sup>	ued				
All Ash Ash On City Tie Cith Arg Phe Arg Glu Ser Thr City Val I Lev Val 210  210  210  211  212  212  213  214  215  216  217  218  218  218  218  219  219  219  219	Thr	Ile	His	Asn		Ser	Gln	His	Asp		Gly	Ser	Tyr	Val		ГÀв			
His Gilu Asm Gily Pro Gily Asm Lyo Thr His Thr Cyc Pro Pro Cyc Pro 225  goa cet gaa etc etg gag gag oeg tea gte tte etc tte eee ea aa Ala Pro Gilu Leu Leu Cyc Ala Leu Leu Chi Pro Pro Lyo 240  goa cet gaa etc etc gag gag oeg tea gte tte etc tte eee ea aa Ala Pro Gilu Leu Leu Cyc Ala Cyc				Gly					Arg					Val			734		
Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys 246 246 257 268 269 260 260 260 260 260 261 265 265 265 265 265 265 265 265 265 265			Asn					Lys					Pro				782		
Pro Lyo App Thr Leu Met 11e Ser Arg Thr Pro Glu Val Thr Cys Val 255 265 265 265 265 265 265 270 265 265 270 265 265 270 265 270 265 265 270 265 270 265 265 270 265 270 275 275 275 280 280 280 280 280 280 280 280 280 280	_	Pro	-		_		Gly	-		-		Leu					830		
Val Val Amp Val Ser Him Glu Amp Pro Glu Val Lym Phe Am Trp Tyr 275  gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg gag gag yal Val Amp Gly Val Glu Val Him Amn Ala Lym Thr Lym Pro Arg Glu Glu Sun Amp Gly Val Glu Val Him Amn Ala Lym Thr Lym Pro Arg Glu Glu Sun Amp Gly Val Glu Val Him Amn Ala Lym Thr Lym Pro Arg Glu Glu Sun Amp Gly Val Glu Val Him Amn Ala Lym Thr Lym Pro Arg Glu Glu Sun Amp Gly Lym Gym Lym Val Leu Him Sun Ala Lym Try Amn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu Him Sun Glu Amp Trp Leu Amn Gly Lym Glu Tyr Lym Gym Lym Val Ser Amn Lym Sun Amn Sun Sun Sun Sun Sun Sun Amn Sun Sun Sun Sun Sun Sun Sun Sun Sun Su	Pro	Lys	-			Met					Pro		-		_	Val	878		
Val Asp Gly Val Glu Val Hie Asp Ala Lys Thr Lys Pro Arg Glu Glu 295 300 205 206 207 207 208 208 208 209 209 209 200 209 200 201 201 201 201 201 201 201 201 201					Ser					Glu					${\tt Trp}$		926		
Chart Tyr Agn Ser Thr Tyr Agn Val Val Ser Val Leu Thr Val Leu Hie 305		_		Val					Āla	_		_	_	Arg			974		
Gin Asp Trp Leu Asn Giy Lys Giu Tyr Lys Cys Lys Val Ser Asn Lys 320  gcc ctc cca gcc ccc atc gag asa acc atc tcc asa gcc asa ggg cag			Asn					Val					Thr				1022		
Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln 3355  345  346  355  347  348  348  349  349  350  348  350  350  360  360  360  365  365  365		Asp					Lys					Lys					1070		
Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu 365  acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa ggc ttc tc ta ccc Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro 370  agc gac atc gcc gtg gag tgg gag agc aat ggg cag ccg gag aac aac aac 1262  Ser Asp Tle Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Bys Gly Ser Asn Gly Gln Pro Bys Gly Ser Asn Gly Gln Pro Gly Ser Phe Phe Leu 400  400  410  410  420  420  420  420	Āla					Ile					Ser					Gln	1118		
Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro 380    agc gac atc gcc gtg gag tgg gag ac aat ggg cag cag cgc gag ac as ggc cgc file Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn 395    tac aag acc acg cct ccc gtg ctg gac tcc gac ggc tcc ttc tc ctc Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu 400    tat agc aag ctc acc gtg gac aag agc agg tgg cag agg ggg acc gtc acc gac ggg acc gtc acc gac ggg acc acc gtc acc gtc Asp Lys Ser Arg Trp Gln Gln Gly Asn Val 410    tat agc aag ctc tcc gtg atg acc agg ggt acc gtg cac acc acc acc acc acc acc acc acc ac					Gln					Pro					Glu		1166		
Ser         Asp         11e         Ala         Val         Glu         Trp         Glu         Ser         Asn         Gly         Glu         Pro         Glu         Asn         Asn         Gly         Asn         Asn <td></td> <td></td> <td></td> <td>Gln</td> <td></td> <td></td> <td></td> <td></td> <td>Cys</td> <td></td> <td></td> <td></td> <td></td> <td>Phe</td> <td></td> <td></td> <td>1214</td> <td></td> <td></td>				Gln					Cys					Phe			1214		
Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu  tat agc aag ctc acc gtg gac aag agc tys Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gln Gly Asn Val 415			Ile					Glu					Pro				1262		
Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val 415  ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac tac acg cag Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln 435  aag agc ctc tcc ctg tct ccg ggt aaa tgaagegece c Lys Ser Leu Ser Leu Ser Pro Gly Lys 450  455  455  4210 SEQ ID NO 14  4211> LENGTH: 455  4212> TYPE: PRT  4213> ORGANISM: Homo sapiens  4400 SEQUENCE: 14  Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser 1 5 10 15  Cys Leu Leu Cu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu		Lys					Val					Gly					1310		
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Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser  1 5 10 15  Cys Leu Leu Leu Thr Gly Ser Ser Gly Gly Arg Pro Phe Val Glu	<21 <21	1> Ll 2> T	ENGTI YPE :	H: 4	55	o sa	pien	S											
1 5 10 15  Cys Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu																			
		Val	Ser	Tyr		Asp	Thr	Gly	Val		Leu	CÀa	Ala	Leu		Ser			
	CÀa	Leu	Leu		Thr	Gly	Ser	Ser		Gly	Gly	Arg	Pro		Val	Glu			

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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	CAa	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
Ile	Gly	Leu	Leu 100	Thr	CÀa	Glu	Ala	Thr 105	Val	Asn	Gly	His	Leu 110	Tyr	Lys
Thr	Asn	Tyr 115	Leu	Thr	His	Arg	Gln 120	Thr	Asn	Thr	Ile	Ile 125	Asp	Ile	Gln
Leu	Leu 130	Pro	Arg	Lys	Ser	Leu 135	Glu	Leu	Leu	Val	Gly 140	Glu	Lys	Leu	Val
Leu 145	Asn	Cys	Thr	Val	Trp 150	Ala	Glu	Phe	Asn	Ser 155	Gly	Val	Thr	Phe	Asp 160
Trp	Asp	Tyr	Pro	Gly 165	Lys	Gln	Ala	Glu	Arg 170	Gly	ГÀа	Trp	Val	Pro 175	Glu
Arg	Arg	Ser	Gln 180	Gln	Thr	His	Thr	Glu 185	Leu	Ser	Ser	Ile	Leu 190	Thr	Ile
His	Asn	Val 195	Ser	Gln	His	Asp	Leu 200	Gly	Ser	Tyr	Val	Сув 205	ГÀз	Ala	Asn
Asn	Gly 210	Ile	Gln	Arg	Phe	Arg 215	Glu	Ser	Thr	Glu	Val 220	Ile	Val	His	Glu
Asn 225	Gly	Pro	Gly	Asp	Lys 230	Thr	His	Thr	CAa	Pro 235	Pro	CAa	Pro	Ala	Pro 240
Glu	Leu	Leu	Gly	Gly 245	Pro	Ser	Val	Phe	Leu 250	Phe	Pro	Pro	ГÀв	Pro 255	Lys
Asp	Thr	Leu	Met 260	Ile	Ser	Arg	Thr	Pro 265	Glu	Val	Thr	Cys	Val 270	Val	Val
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Trp	Leu	Asn	Gly	Lys 325	Glu	Tyr	ГÀз	Сув	330 FÀa	Val	Ser	Asn	ГÀз	Ala 335	Leu
Pro	Ala	Pro	Ile 340	Glu	Lys	Thr	Ile	Ser 345	Lys	Ala	Lys	Gly	Gln 350	Pro	Arg
Glu	Pro	Gln 355	Val	Tyr	Thr	Leu	Pro 360	Pro	Ser	Arg	Asp	Glu 365	Leu	Thr	Lys
Asn	Gln 370	Val	Ser	Leu	Thr	Сув 375	Leu	Val	ГÀа	Gly	Phe 380	Tyr	Pro	Ser	Asp
Ile 385	Ala	Val	Glu	Trp	Glu 390	Ser	Asn	Gly	Gln	Pro 395	Glu	Asn	Asn	Tyr	Lys 400
Thr	Thr	Pro	Pro	Val 405	Leu	Asp	Ser	Asp	Gly 410	Ser	Phe	Phe	Leu	Tyr 415	Ser
Lys	Leu	Thr	Val 420	Asp	Lys	Ser	Arg	Trp 425	Gln	Gln	Gly	Asn	Val 430	Phe	Ser
CAa	Ser	Val 435	Met	His	Glu	Ala	Leu 440	His	Asn	His	Tyr	Thr 445	Gln	Lys	Ser
Leu	Ser	Leu	Ser	Pro	Gly	Lys									

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		cgg gtt acg tca cct aa Arg Val Thr Ser Pro As 60	
		gac act ttg atc cct ga Asp Thr Leu Ile Pro As 75	
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		tgt gaa gca aca gtc aa Cys Glu Ala Thr Val As 105	
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		tct ggg agt gag atg aa Ser Gly Ser Glu Met Ly 185	
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											-	con	tin	ued			
gtg q Val V																	
tac q Tyr V																912	
						tac Tyr											
cac ( His (																1008	
aaa q Lys <i>I</i>																	
cag ( Gln I																	
ctg a Leu :																1152	
ccc a Pro S 385																	
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ctc t Leu '																	
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Phe V	Val	Glu 35	Met	Tyr	Ser	Glu	Ile 40	Pro	Glu	Ile	Ile	His 45	Met	Thr	Glu		
	50					55	-				60						
Val '			-	-	70			Ī		75			Ī	Ī	80		
Arg :	Ile	Ile	Trp	85 85	Ser	Arg	Lys	Gly	Phe 90	Ile	Ile	Ser	Asn	Ala 95	Thr		
Tyr I	-		100	-				105					110	_			
Leu :	Tyr	Lys 115	Thr	Asn	Tyr	Leu	Thr 120	His	Arg	Gln	Thr	Asn 125	Thr	Ile	Ile		

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Asp Val Val Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu 130 135 140

Lys Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile 145 150 160

85

Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu 165 \$170\$

Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe 180  $\,$  180  $\,$  180  $\,$ 

Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu 195  $\phantom{\bigg|}200\phantom{\bigg|}$ 

Phe Val Arg Val His Glu Lys Asp Lys Thr His Thr Cys Pro Pro Cys 225  $\phantom{\bigg|}230\phantom{\bigg|}$  230  $\phantom{\bigg|}240\phantom{\bigg|}$ 

Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro 245 250 255

Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys  $260 \hspace{1cm} 265 \hspace{1cm} 270 \hspace{1cm}$ 

Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu 290 295 300

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu 305 310 315 320

His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn 325 330 335

Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly 340 345 350

Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu 355 \$360\$

Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr

Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn 385 \$390\$

Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe 405 \$410\$

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Asp	Gly 50	Lys	Arg	Ile	Ile	Trp 55	Asp	Ser	Arg	Lys	Gly 60	Phe	Ile	Ile	Ser
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Thr	Ile	Ile	Asp 100	Val	Val	Leu	Ser	Pro 105	Ser	His	Gly	Ile	Glu 110	Leu	Ser
Val	Gly	Glu 115	Lys	Leu	Val	Leu	Asn 120	CÀa	Thr	Ala	Arg	Thr 125	Glu	Leu	Asn
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Lys	Lys	Phe	Leu	Ser 165	Thr	Leu	Thr	Ile	Asp 170	Gly	Val	Thr	Arg	Ser 175	Asp
Gln	Gly	Leu	Tyr 180	Thr	CAa	Ala	Ala	Ser 185	Ser	Gly	Leu	Met	Thr 190	Lys	rya
Asn	Ser	Thr 195	Phe	Val	Arg	Val	His 200	Glu	Lys	Gly	Pro	Gly 205	Asp	Lys	Thr
His	Thr 210	Сув	Pro	Pro	Cys	Pro 215	Ala	Pro	Glu	Leu	Leu 220	Gly	Gly	Pro	Ser
Val 225	Phe	Leu	Phe	Pro	Pro 230	Lys	Pro	Lys	Asp	Thr 235	Leu	Met	Ile	Ser	Arg 240
Thr	Pro	Glu	Val	Thr 245	CÀa	Val	Val	Val	Asp 250	Val	Ser	His	Glu	Asp 255	Pro
Glu	Val	Lys	Phe 260	Asn	Trp	Tyr	Val	Asp 265	Gly	Val	Glu	Val	His 270	Asn	Ala
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Lys 305	Cys	Lys	Val	Ser	Asn 310	Lys	Ala	Leu	Pro	Ala 315	Pro	Ile	Glu	Lys	Thr 320
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Pro	Pro	Ser	Arg 340	Asp	Glu	Leu	Thr	Lys 345	Asn	Gln	Val	Ser	Leu 350	Thr	Cya
Leu	Val	155 355	Gly	Phe	Tyr	Pro	Ser 360	Asp	Ile	Ala	Val	Glu 365	Trp	Glu	Ser
Asn	Gly 370	Gln	Pro	Glu	Asn	Asn 375	Tyr	Lys	Thr	Thr	Pro 380	Pro	Val	Leu	Asp
Ser 385	Asp	Gly	Ser	Phe	Phe 390	Leu	Tyr	Ser	rys	Leu 395	Thr	Val	Asp	ГÀа	Ser 400
Arg	Trp	Gln	Gln	Gly 405	Asn	Val	Phe	Ser	Cys 410	Ser	Val	Met	His	Glu 415	Ala
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What is claimed is:

- 1. A cell culture composition comprising cells suspended in culture medium, wherein the cells contain an expression vector comprising a nucleic acid molecule that encodes the amino acid sequence of Flt1D2.Flk1D3.FcΔC1(a) (SEQ ID NO:12) or VEGFR1R2-FcΔC1(a) (SEQ ID NO:16).
- 2. The cell culture composition of claim 1, wherein the cells are selected from the group consisting of bacterial cells, yeast cells, insect cells and mammalian cells.
- 3. The cell culture composition of claim 2, wherein the cells are *E. coli* cells, COS cells or CHO cells.
- **4**. The cell culture composition of claim **3**, wherein the cells are CHO cells.
- 5. The cell culture composition of claim 1, wherein the culture medium is a glutamine-free medium.

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- **6**. The cell culture composition of claim **1**, wherein the culture medium contains 5% fetal bovine serum.
- 7. The cell culture composition of claim 1, wherein the culture medium has a pH of 7.2.
- 8. The cell culture composition of claim 1 contained within a roller bottle.
- $\boldsymbol{9}.$  The cell culture composition of claim  $\boldsymbol{1}$  contained within a bioreactor.
- 10. The cell culture composition of claim 9, wherein the
- 11. The cell culture composition of claim 9, wherein the bioreactor is a 40 L bioreactor.
- 12. The cell culture composition of claim 1, wherein the density of the cells in the culture medium is  $4\times10^6$  cells/mL.

\* \* \* \* \*