

(12) United States Patent

Papadopoulos et al.

(54) MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES

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- (*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 489 days.

This patent is subject to a terminal disclaimer.

- (21) Appl. No.: 11/016,097
- (22) Filed: Dec. 17, 2004

(65)**Prior Publication Data**

US 2005/0163798 A1 Jul. 28, 2005

Related U.S. Application Data

- (62) 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.

A61K 38/18	(2006.01)
C07K 14/71	(2006.01)
C12N 15/62	(2006.01)

- (52) U.S. Cl. 424/134.1; 424/192.1; 514/2; 514/12; 530/350; 536/23.4
- Field of Classification Search None (58)See application file for complete search history.

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ABSTRACT (57)

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

7 Claims, 55 Drawing Sheets











Fig.6B.









Fig.10A.

10 50 60 20 30 40 * ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC TAC CAG TOG ATG ACC CTG TGG COC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu> 70 100 110 120 80 90 * ACA GGA TCT AGT TCA GGT TCA AAA TTA AAA GAT CCT GAA CTG AGT TTA AAA GGC ACC CAG TGT CCT AGA TCA AGT CCA AGT TTT AAT TTT CTA GGA CTT GAC TCA AAT TTT CCG TGG GTC Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro Glu Leu Ser Leu Lys Gly Thr Gln> 160 170 180 130 140 150 * * CAC ATC ATG CAA GCA GGC CAG ACA CTG CAT CTC CAA TGC AGG GGG GAA GCA GCC CAT AAA GTG TAG TAC GTT CGT CCG GTC TGT GAC GTA GAG GTT ACG TCC CCC CTT CGT CGG GTA TTT His Ile Met Gln Ala Gly Gln Thr Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys> 190 200 210 220 230 240 · • * + * ÷ TIG TET TTG CET GAA ATG GTG AGT AAG GAA AGC GAA AGG ETG AGC ATA ACT AAA TET GCE ACC AGA AAC GGA CTT TAC CAC TCA TTC CTT TCG CTT TCC GAC TCG TAT TGA TTT AGA CGG Trp Ser Leu Pro Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala> 250 260 270 280 290 300 * ٠ * * * TGT GGA AGA AAT GGC AAA CAA TTC TGC AGT ACT TTA ACC TTG AAC ACA GCT CAA GCA AAC ACA CCT TCT TTA CCG TTT GTT AAG ACG TCA TGA AAT TGG AAC TTG TGT CGA GTT CGT TTG Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr Ala Gln Ala Asn> 340 350 360 310 320 330 CAC ACT GGC TTC TAC AGC TGC AAA TAT CTA GCT GTA CCT ACT TCA AAG AAG AAG GAA ACA GTG TGA CCG AAG ATG TCG ACG TTT ATA GAT CGA CAT GGA TGA AGT TTC TTC TTC CTT TGT His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val Pro Thr Ser Lys Lys Glu Thr> 390 400 410 420 370 380 * GAA TCT GCA ATC TAT ATA TTT ATT AGT GAT ACA GGT AGA CCT TTC GTA GAG ATG TAC AGT CTT AGA CGT TAG ATA TAT AAA TAA TCA CTA TGT CCA TCT GGA AAG CAT CTC TAC ATG TCA Glu Ser Ala Ile Tyr Ile Phe Ile Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser> 460 470 480 430 440 450 GAA ATC CCC GAA ATT ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT CTT TAG GGG CTT TAA TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val> 540 490 500 510 520 530 ACG TCA CCT AAC ATC ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT TGC AGT GGA TTG TAG TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp> Fig.10B.

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ССТ	TTT	GCG	TAT	TAG	ACC	CTG	TCA	тст	TTC	CCG	AAG	TAG	тат	AGT	TTA	CGT	TGC	ATG	TTT
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Lys	Leu	Leu	Arg	Gly	His	Thr	Leu	Val	Leu	Asn	Cys	Thr	Ala	Thr	Thr	Pro	Leu	Asn	Thr>
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TCT	CAA	GTT	TAC	TCC	ACC	TCA	ATG	GGA	CTA	CTT	TTT	TTA	TTC	TCT	CGA	AGG	CAT	TCC	GCT
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CGA	* ATT	85 GAC	50 * CAA	* AGC	AAT	360 * TCC	CAT	* GCC	870 * AAC	ATA	* TTC	BE	BO * AGT	* GTT	CTT	890 * ACT	ATT	* GAC	900 * AAA
CGA GCT	* ATT TAA	85 GAC CTG	50 * CAA GTT	* AGC TCG	AAT TTA	360 * TCC AGG	CAT GTA	* GCC CGG	870 * AAC TTG	ATA TAT	* TTC AAG	88 TAC ATG	BO * AGT TCA	* GTT CAA	CTT GAA	ACT TGA	атт Таа	* GAC CTG	900 * AAA TTT
CGA GCT Arg	* ATT TAA Ile	85 GAC CTG Asp	50 * CAA GTT Gln	* AGC TCG Ser	AAT TTA Asn	360 * TCC AGG Ser	CAT GTA His	* GCC CGG Ala	870 * AAC TTG Asn	ATA TAT Ile	* TTC AAG Phe	BE TAC ATG Tyr	* AGT TCA Ser	* GTT CAA Val	CTT GAA Leu	390 * ACT TGA Thr	ATT TAA Ile	* GAC CTG Asp	900 * AAA TTT Lys>
CGA GCT Arg	* ATT TAA Ile	85 GAC CTG Asp	50 * CAA GTT Gln	* AGC TCG Ser	AAT TTA Asn	360 * TCC AGG Ser	CAT GTA His	* GCC CGG Ala	870 * AAC TTG Asn	ATA TAT Ile	* TTC AAG Phe	BE TAC ATG Tyr	30 * AGT TCA Ser	* GTT CAA Val	CTT GAA Leu	390 * ACT TGA Thr	ATT TAA Ile	* GAC CTG Asp	900 * AAA TTT Lys>
CGA GCT Arg	* ATT TAA Ile	85 GAC CTG Asp 91	CAA GTT Gln	* AGC TCG Ser	AAT TTA Asn	360 * TCC AGG Ser 20	CAT GTA His	* GCC CGG Ala	870 * AAC TTG Asn 930	ATA TAT Ile	* TTC AAG Phe	BE TAC ATG Tyr 94	AGT TCA Ser	* GTT CAA Val	CTT GAA Leu	ACT TGA Thr	ATT TAA Ile	* GAC CTG ASP	900 * AAA TTT Lys> 960
CGA GCT Arg	* ATT TAA Ile *	85 GAC CTG Asp 91	CAA GTT GIn 10	* AGC TCG Ser *	AAT TTA Asn	360 * TCC AGG Ser 20 *	CAT GTA His	* GCC CGG Ala *	870 * AAC TTG Asn 930 *	ATA TAT Ile	* TTC AAG Phe	BE TAC ATG Tyr 94	30 * AGT TCA Ser 40 *	* GTT CAA Val	CTT GAA Leu	ACT TGA Thr 50	ATT TAA Ile	* GAC CTG Asp	900 * AAA TTT Lys> 960 *
CGA GCT Arg ATG	* ATT TAA Ile * CAG	85 GAC CTG Asp 91 AAC	CAA GTT GIn 10 * AAA	* AGC TCG Ser * GAC	AAT TTA Asn	360 * TCC AGG Ser 920 * GGA	CAT GTA His CTT	* GCC CGG Ala * TAT	870 * AAC TTG Asn 930 * ACT	ATA TAT Ile TGT	* TTC AAG Phe * CGT	BE TAC ATG Tyr 94 GTA	AGT TCA Ser 10 * AGG	* GTT CAA Val * AGT	CTT GAA Leu GGA	ACT TGA Thr 950 * CCA	ATT TAA Ile TCA	* GAC CTG Asp * TTC	900 * AAA TTT Lys> 960 * AAA
CGA GCT Arg ATG TAC	* ATT TAA Ile * CAG GTC	85 GAC CTG Asp 91 AAC TTG	CAA GTT GIN 0 * AAA TTT	* AGC TCG Ser * GAC CTG	AAT TTA Asn S AAA TTT	360 * TCC AGG Ser 20 * GGA CCT	CAT GTA His CTT GAA	* GCC CGG Ala * TAT	870 * AAC TTG Asn 930 * ACT TGA	ATA TAT Ile TGT ACA	* TTC AAG Phe * CGT GCA	BE TAC ATG Tyr 94 GTA CAT	AGT TCA Ser 10 * AGG TCC	* GTT CAA Val * AGT TCA	CTT GAA Leu GGA CCT	ACT TGA Thr 50 * CCA GGT	ATT TAA Ile TCA AGT	* GAC CTG Asp * TTC	900 * AAA TTT Lys> 960 * AAA TTT
CGA GCT Arg ATG TAC Met	* ATT TAA Ile * CAG GTC GIn	85 GAC CTG Asp 91 AAC TTG Asn	CAA GTT GIn LO * AAA TTT LVS	* AGC TCG Ser * GAC CTG Asp	AAT TTA Asn AAA TTT Lvs	360 * TCC AGG Ser 20 * GGA CCT Glv	CAT GTA His CTT GAA Leu	* GCC CGG Ala * TAT ATA TVT	870 * AAC TTG Asn 930 * ACT TGA Thr	ATA TAT Ile TGT ACA Cys	* TTC AAG Phe * CGT GCA Arg	BE TAC ATG Tyr 94 GTA CAT Val	AGT TCA Ser 40 * AGG TCC Arg	* GTT CAA Val * AGT TCA Ser	CTT GAA Leu GGA CCT GIY	390 * ACT TGA Thr 50 * CCA GGT Pro	ATT TAA Ile TCA AGT Ser	* GAC CTG Asp * TTC AAG Phe	AAA TTT Lys> 960 * AAA TTT Lys>
CGA GCT Arg ATG TAC Met	* ATT TAA Ile * CAG GTC GIn	8 GAC CTG Asp 91 AAC TTG Asn	CAA GTT Gln 0 * AAA TTT Lys	* AGC TCG Ser * GAC CTG Asp	AAT TTA Asn S AAA TTT Lys	360 * TCC AGG Ser 20 * GGA CCT Gly	CAT GTA His CTT GAA Leu	* CGG Ala * TAT ATA Tyr	870 * AAC TTG Asn 930 * ACT TGA Thr	ATA TAT Ile TGT ACA Cys	* TTC AAG Phe * CGT GCA Arg	BE TAC ATG Tyr 94 GTA CAT Val	AGT TCA Ser 10 * AGG TCC Arg	* GTT CAA Val * AGT TCA Ser	CTT GAA Leu GGA CCT Gly	390 * ACT TGA Thr 550 * CCA GGT Pro	ATT TAA Ile TCA AGT Ser	* GAC CTG Asp * TTC AAG Phe	AAA TTT Lys> 960 * AAA TTT Lys>
CGA GCT Arg ATG TAC Met	* TAA Ile * CAG GTC Gln	85 GAC CTG Asp 91 AAC TTG Asn	CAA GTT GIn CIN AAA TTT Lys	* AGC TCG Ser * GAC CTG Asp	AAT TTA Asn AAA TTT Lys	360 * TCC AGG Ser 920 * GGA CCT Gly 980	CAT GTA His CTT GAA Leu	* CGG Ala * TAT ATA Tyr	870 * AAC TTG Asn 930 * ACT TGA Thr 990	ATA TAT Ile TGT ACA Cys	* TTC AAG Phe * CGT GCA Arg	BE TAC ATG Tyr 94 GTA CAT Val 100	AGT TCA Ser 10 * AGG TCC Arg	* GTT CAA Val * AGT TCA Ser	CTT GAA Leu GGA CCT Gly	ACT TGA Thr 550 * CCA GGT Pro	ATT TAA Ile TCA AGT Ser	* GAC CTG Asp * TTC AAG Phe	AAA TTT Lys> 960 * AAA TTT Lys>
CGA GCT Arg ATG TAC Met	* ATT TAA Ile * CAG GTC Gln *	85 GAC CTG Asp 91 AAC TTG Asn 97	CAA GTT Gln 10 * AAA TTT Lys 70	* AGC TCG Ser * GAC CTG Asp	AAT TTA Asn S AAA TTT Lys	360 * TCC AGG Ser 20 * GGA CCT Gly 80 *	CAT GTA His CTT GAA Leu	* GCC CGG Ala * TAT ATA Tyr	870 * AAC TTG Asn 930 * ACT TGA Thr 990	ATA TAT Ile TGT ACA Cys	* TTC AAG Phe * CGT GCA Arg	BE TAC ATG Tyr 94 GTA CAT Val 100	AGT TCA Ser 40 * AGG TCC Arg	* GTT CAA Val * AGT TCA Ser *	CTT GAA Leu GGA CCT Gly 1	ACT TGA Thr 550 * CCA GGT Pro 010	ATT TAA Ile TCA AGT Ser	* GAC CTG Asp * TTC AAG Phe	900 * AAA TTT Lys> 960 * AAA TTT Lys> 1020
CGA GCT Arg ATG TAC Met	* ATT TAA Ile * CAG GTC GIn *	85 GAC CTG Asp 91 AAC TTG Asn 97	CAA GTT Gln LO * AAA TTT Lys 70	* AGC TCG Ser * GAC CTG Asp	AAT TTA Asn AAA TTT Lys	360 * TCC AGG Ser 220 * GGA CCT Gly 80 *	CAT GTA His CTT GAA Leu	* GCC CGG Ala * TAT ATA Tyr *	870 * AAC TTG Asn 930 * ACT TGA Thr 990 *	ATA TAT Ile TGT ACA Cys	* TTC AAG Phe * CGT GCA Arg *	BE TAC ATG Tyr 94 GTA CAT Val 100	AGT TCA Ser 40 * AGG TCC Arg	* GTT CAA Val * AGT TCA Ser *	CTT GAA Leu GGA CCT Gly 1 GAG	ACT TGA Thr 50 * CCA GGT Pro 010	ATT TAA Ile TCA AGT Ser	* GAC CTG Asp * TTC AAG Phe	900 * AAA TTT Lys> 960 * AAA TTT Lys> 1020 *
CGA GCT Arg ATG TAC Met	* ATT TAA Ile * CAG GTC GIn * GTT	85 GAC CTG Asp 91 AAC TTG Asn 97 AAC	CAA GTT GIn LO AAA TTT Lys ACC	* AGC TCG Ser * GAC CTG Asp * TCA	AAT TTA Asn AAA TTT Lys GTG	360 * TCC AGG Ser 220 * GGA CCT Gly 80 * CAT	CAT GTA His CTT GAA Leu ATA	* GCC CGG Ala * TAT TAT * TAT * TAT	870 * AAC TTG ASN 930 * ACT TGA Thr 990 * GAT	ATA TAT Ile TGT ACA Cys	* TTC AAG Phe * CGT GCA Arg * GCA	BE TAC ATG Tyr 94 GTA CAT Val 100 GGC	AGT TCA Ser 40 * AGG TCC Arg	* GTT CAA Val * AGT TCA Ser * GGC	GGA GGA CCT GIY 1 GAG	ACT TGA TGA Thr 50 * CCA GGT Pro 010 * CCC	ATT TAA Ile TCA AGT Ser AAA	* GAC CTG Asp * TTC AAG Phe *	900 * AAA TTT Lys> 960 * AAA TTT Lys> 1020 * TGT
CGA GCT Arg ATG TAC Met TCT AGA	* ATT TAA Ile * CAG GTC Gln * GTT CAA	8: GAC CTG Asp 91 AAC TTG ASn 9' AAC TTG	50 * CAA GTT Gln 10 * AAA TTT Lys 70 * ACC TGG	* AGC TCG Ser * GAC CTG Asp * TCA AGT	AAT TTA Asn AAA TTT Lys GTG CAC	360 * TCC AGG Ser 220 * GGA CCT GLY 980 * CAT GTA Utic	CAT GTA His CTT GAA Leu ATA	* GCC CGG Ala * TAT TAT ATA ATA	870 * AAC TTG Asn 930 * ACT TGA Thr 990 * GAT CTA	ATA TAT Ile TGT ACA Cys AAA TTT	* TTC AAG Phe * CGT GCA Arg * GCA CGT	BE TAC ATG Tyr 94 GTA CAT Val 100 GGC CCG CCG	AGT TCA Ser 40 * AGG TCC Arg 00 * CCG GGC	* GTT CAA Val AGT TCA Ser * GGC CCG	CTTT GAA Leu GGA GCTT Gly 1 GAG CTCC	ACT TGA TGA Thr 550 * CCA GGT Pro 010 * CCC GGG	ATT TAA Ile TCA AGT Ser AAA TTT	* GAC CTG Asp * TTC AAG Phe * TCT AGA	900 * AAA TTT Lys> 960 * AAA TTT Lys> 1020 * TGT ACA
CGA GCT Arg ATG TAC Met TCT AGA Ser	* ATT TAA Ile * CAG GTC Gln * CAA Val	85 GAC CTG Asp 91 AAC TTG Asn 97 AAC TTG Asn	CAA GTT Gln L0 * AAA TTT Lys ACC TGG Thr	* AGC Ser & GAC CTG Asp * TCA AGT Ser	AAT TTA Asn AAA TTT Lys GTG CAC Val	360 * TCC AGG Ser 920 * GGA CCT Gly 980 * CAT GTA His	CAT GTA His CTT GAA Leu ATA TAT Ile	* GCC CGG Ala * TAT TAT ATA ATA TYT	870 * AAC TTG Asn 930 * ACT TGA Thr 930 * GAT CTA Asp	ATA TAT Ile TGT ACA Cys AAA TTT Lys	* TTC AAG Phe CGT GCA Arg & CGT Ala	BE TAC ATG Tyr 94 GTA CAT Val 100 GGC CCG Gly	30 * AGT TCA Ser 40 * AGG TCC Arg 00 * CCG GGC Pro	* GTT CAA Val * AGT TCA Ser * GGC GGC Gly	GGA GGA CCTT Gly 1 GAG CCTC Gly CCTC Glu	ACT TGA TGA Thr 950 * CCA GGT Pro 010 * CCC GGG Pro	ATT TAA Ile TCA AGT Ser AAA TTT Lys	* GAC CTG Asp * TTC AAG Phe * TCT AGA Ser	900 * AAA TTT Lys> 960 * AAA TTT Lys> 1020 * TGT ACA Cys>
CGA GCT Arg ATG TAC Met TCT AGA Ser	* ATT TAA Ile * CAG GTC Gln * GTT CAA Val	85 GAC CTG Asp 91 AAC TTG Asn 97 AAC TTG Asn	CAA GTT Gln L0 * AAA TTT Lys ACC TGG Thr	* AGC Ser * GAC CTG Asp * TCA AGT Ser	AAT TTA Asn AAA TTT Lys GTG GTG CAC Val	360 * TCC AGG Ser 920 * GGA CCT Gly 980 * CAT GTA His	CAT GTA His CTT GAA Leu ATA TAT Ile	* GCC CGG Ala * TAT ATA Tyr * TAT ATA Tyr	870 * AAC TTG Asn 930 * ACT TGA Thr 990 * GAT Asp	ATA TAT Ile TGT ACA Cys AAA TTT Lys	* TTC AAG Phe * CGT Arg GCA CGT Ala	SE TAC ATG Tyr 94 GTA CAT 100 GGC CCG Gly	AGT TCA Ser AGT AGG AGG AGG CCG GGC Pro	* GTT CAA Val * AGT TCA Ser * GGC CGG Gly	GGA CCTT GAA Leu GGA CCT Gly 1 GAG CTC Glu	ACT TGA TGA Thr 950 * CCA GGT Pro 010 * CCC GGG Pro	ATT TAA Ile TCA AGT Ser AAA TTT Lys	* GAC CTG ASp * TTC AAG Phe * TCT AGA Ser	900 * AAA TTT Lys> 960 * AAA TTT Lys> 1020 * TGT ACA Cys>
CGA GCT Arg ATG TAC Met TCT AGA Ser	* ATT TAA Ile * CAG GTC GIn * GTT CAA Val	8: GAC CTG Asp 91 AAC TTG Asn 9' AAC TTG Asn 10	CAA GTT Gln L0 * AAA TTT Lys 70 * ACC TGG Thr 30	* AGC Ser GAC CTG Asp * TCA AGT Ser	AAT TTA Asn AAA TTT Lys GTG GTG CAC Val	360 * TCC AGG Ser 920 * GGA CCT Gly 980 * CAT GTA His 040	CAT GTA His CTT GAA Leu ATA TAT Ile	* GCC CGG Ala * TAT ATA Tyr * TAT ATA Tyr	870 * AAC TTG ASN 930 * ACT TGA TGA TGA CTA ASP 10500	ATA TAT Ile TGT ACA Cys AAA TTT Lys	* TTC AAG Phe * CGT GCA Arg & CGA CGT Ala	BE TAC ATG Tyr 94 GTA CAT Val 100 GGC CCG Gly 10	AGT TCA Ser TCA AGG TCC Arg 00 * CCG GGC Pro 60	* GTT CAA Val * AGT TCA Ser * GGC CGG Gly	CTT GAA Leu GGA CCT Gly 1 GAG CTC Glu 1	ACT TGA Thr 550 * CCA GGT Pro 010 * CCC GGG Pro 070	ATT TAA Ile TCA AGT Ser AAA TTT Lys	* GAC CTG Asp * TTC AAG Phe * TCT AGA Ser	900 * AAA TTT Lys> 960 * AAA TTT Lys> 1020 * TGT ACA Cys> 1080
CGA GCT Arg ATG TAC Met TCT AGA Ser	* ATT TAA Ile * CAG GTC Gln * CAA Val *	8: GAC CTG Asp 91 AAC TTG Asn 9° AAC TTG Asn 10	CAA GTT Gln L0 * AAA TTT Lys 70 * ACC TGG Thr 30 *	* AGC TCG Ser * GAC CTG Asp * TCA AGT Ser *	AAT TTA Asn AAA TTT Lys GTG CAC Val 1	TCC AGG Ser 220 * GGA CCT Gly 980 * CAT GTA His 040	CAT GTA His CTT GAA Leu ATA TAT Ile	* GCC CGG Ala * TAT TAT ATA Tyr * TAT ATA Tyr	870 * AAC TTG Asn 930 * ACT TGA Thr 9900 * GAT CTA Asp 10500 *	ATA TAT Ile TGT ACA Cys AAA TTT Lys	* TTIC AAG Phe CGT CGT AIg CGT Ala	886 TAC ATG Tyr 94 GTA CAT Val 100 GGC CCG Gly 100	30 * AGT TCA Ser 10 * AGG TCC Arg 00 * CCG GGC Pro 60	* GTT CAA Val * AGT TCA Ser * GGC CCG Gly	CTT GAA Leu GGA CCT Gly 1 GAG CTC Glu 1	390 * ACT TGA Thr 950 * CCA GGT Pro 010 * CCC GGG Pro 070 *	ATT TAA Ile TCA AGT Ser AAA TTT Lys	* GAC CTG Asp * TTC AAG Phe * TCT AGA Ser *	900 * AAA TTT Lys> 960 * AAA TTT Lys> 1020 * TGT ACA Cys> 1080 *
CGA GCT Arg ATG TAC Met TCT AGA Ser GAC	* ATT TAA Ile * CAG GTC GIn * CAA Val * AAA	8: GAC CTG Asp 91 AAC TTG Asn 9' AAC TTG Asn 10: ACT	50 * CAA GIT GIN * AAA TTT Lys * ACC TGG Thr 30 * CAC	* AGC Ser * GAC CTG Asp * TCA AGT Ser * ACA	AAT TTA Asn S AAA TTT Lys GTG CAC Val 1 1	360 * TCC AGG Ser 920 * GGA CCT Gly 980 * CAT GTA His 040 *	CAT GTA His CTT GAA Leu ATA TAT Ile CCG	* GCC CGG Ala * TAT TAT ATA TYT * TAT ATA Tyr	870 * AAC TTG Asn 930 * ACT TGA TTGA Thr 990 * GAT CTA Asp 10500 *	ATA TAT Ile TGT ACA Cys AAA TTT Lys GCA	* TTIC AAG Phe CGT GCA Arg GCA CGT Ala , CCT	886 TAC ATG Tyr 94 GTA CAT Val 100 GGC CCG Gly 100 GAA	30 * AGT TCA Ser 10 * AGG TCC Arg 00 * CCG GGC Pro 60 * CTC	* GTT CAA Val * AGT TCA Ser * GGC CCG Gly * CTG	CTT GAA Leu GGA CCT Gly 1 GAG CTC Glu 1 CCC Glu	ACT TGA TGA Thr 550 * CCA GGT Pro 010 * CCC GGG Pro 070 *	ATT TAA Ile TCA AGT Ser AAA TTT Lys	* GAC CTG Asp * TTC AAG Phe * TCT AGA Ser * TCA	900 * AAA TTT Lys> 960 * AAA TTT Lys> 1020 * TGT ACA Cys> 1080 * GTC

Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val>

## Fig.10C.

		109	0		11	00		1	110			112	0		11	30		1	140
	*		*	*		*		*	*		*		*	*		*		*	*
TIC	CIC	TTC	CCC	CCA	AAA	CCC	AAG	GAC	ACC	CIC	ATG	ATC	TCC	CGG	ACC	CCT	GAG	GTC	ACA
AAG	GAG	aag	GGG	GGT	TTT	GGG	TIC	CTG	TGG	GAG	TAC	TAG	AGG	GCC	TGG	GGA	CIC	CAG	TGT
Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr>
		115	50		11	.60		1	170			118	80		11	90		1	200
	*		*	*		*		*	*		*		*	*		*		*	*
TGC	GTG	GTG	GTG	GAC	GTG	AGC	CAC	GAA	GAC	CCT	GAG	GTC	AAG	TTC	AAC	TGG	TAC	GTG	GAC
ACG	CAC	CAC	CAC	CTG	CAC	TCG	ĠTG	CTT	CTG	GGA	CTC	CAG	TTC	AAG	TTG	ACC	ATG	CAC	CTG
Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp>
		12:	10		12	220		-	1230			124	10		1:	250		3	260
	*		*	*		*		*	*		*		*	*		*		*	*
GGC	GTG	GAG	GTG	CAT	AAT	GCC	AAG	ACA	AAG	CCG	CGG	GAG	GAG	CAG	TAC	AAC	AGC	ACG	TAC
CCG	CAC	CTC	CAC	GTA	TTA	CGG	TTC	TGT	TTC	GGC	GCC	CTC	CTC	GTC	ATG	TTG	TCG	TGC	ATG
Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr>
		12	70		1:	280			1290			13	00		1	310			1320
	*		*	*	_	*		*	*		*		*	*		*		*	*
CGT	GTG	GTC	AGC	GTC	CTC	ACC	GTC	CTG	CAC	CAG	GAC	TGG	CTG	AAT	GGC	AAG	GAG	TAC	AAG
GCA	CAC	CAG	TCG	CAG	GAG	TGG	CAG	GAC	GTG	GIC	CTG	ACC	GAC	TTA	CCG	TTC	CTC	ATG	TTC
Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys>
		133	30		13	340			1350			130	50		1	370			1380
	*	133	30 *	*	13	340 *		*	1350 *		*	130	50 *	*	1	370 *		*	L380 *
TGC	* AAG	13: GIC	30 * TCC	* AAC	13 AAA	340 * GCC	CTC	* CCA	1350 * GCC	ccc	* ATC	13) GAG	50 * AAA	* ACC	1 ATC	370 * TCC	ала	* GCC	1380 * AAA
TGC ACG	* AAG TTC	13: GTC CAG	30 * TCC AGG	* AAC TTG	13 AAA TTT	340 * GCC CGG	CTC GAG	* CCA GGT	L350 * GCC CGG	CCC GGG	* ATC TAG	13 GAG CTC	50 * AAA TTT	* ACC TGG	1: ATC TAG	370 * TCC AGG	aaa TTT	: * 6CC CGG	1380 * AAA TTT
TGC ACG Cys	* AAG TTC Lys	13: GTC CAG Val	30 * TCC AGG Ser	* AAC TTG Asn	13 AAA TTT Lys	40 * GCC CGG Ala	CTC GAG Leu	* CCA GGT Pro	L350 * GCC CGG Ala	CCC GGG Pro	* ATC TAG Ile	13 GAG CTC Glu	50 * AAA TTT Lys	* ACC TGG Thr	1: ATC TAG Ile	370 * TCC AGG Ser	AAA TTT Lys	* GCC CGG Ala	1380 * AAA TTT Lys>
TGC ACG Cys	* AAG TTC Lys	13: GTC CAG Val	30 * TCC AGG Ser 90	* AAC TTG Asn	13 AAA TTT Lys	GCC CGG Ala	CTC GAG Leu	* CCA GGT Pro	1350 * GCC CGG Ala 1410	CCC GGG Pro	* ATC TAG Ile	130 GAG CTC Glu 14	50 * AAA TTT Lys 20	* ACC TGG Thr	1 ATC TAG Ile	370 * TCC AGG Ser 430	AAA TTT Lys	* GCC CGG Ala	1380 * AAA TTT Lys> 1440
TGC ACG Cys	* AAG TTC Lys	13: GTC CAG Val 13:	30 * TCC AGG Ser 90 *	* AAC TTG Asn	13 AAA TTT Lys 1	GCC CGG Ala 400	CTC GAG Leu	* CCA GGT Pro	1350 * GCC CGG Ala 1410 *	CCC GGG Pro	* ATC TAG Ile	13 GAG CTC Glu 14	50 * AAA TTT Lys 20 *	* ACC TGG Thr	1 ATC TAG Ile 1	370 * TCC AGG Ser 430 *	AAA TTT Lys	* GCC CGG Ala	1380 * AAA TTT Lys> 1440 *
TGC ACG Cys GGG	* AAG TTC Lys * CAG	13: GTC CAG Val 13: CCC	30 * TCC AGG Ser 90 *	* AAC TTG Asn * GAA	13 AAA TTT Lys 14 CCA	GCC CGG Ala 400 * CAG	CTC GAG Leu GTG	* CCA GGT Pro * TAC	1350 * GCC CGG Ala 1410 *	CCC GGG Pro	* ATC TAG Ile *	130 GAG CTC Glu 14 CCA	50 * AAA TTT Lys 20 *	* ACC TGG Thr *	1: ATC TAG Ile 1 GAT	370 * TCC AGG Ser 430 * GAG	AAA TTT Lys CTG	* GCC CGG Ala * ACC	1380 * AAA TTT Lys> 1440 * AAG
TGC ACG Cys GGG CCC	* TTC Lys * CAG GTC	13: GTC CAG Val 13: CCC GGG	30 * TCC AGG Ser 90 * CGA GCT	* AAC TIG Asn * GAA CTT	13 AAA TTT Lys 14 CCA GGT	GCC CGG Ala 400 * CAG GTC	CTC GAG Leu GTG CAC	* GGT Pro * TAC ATG	GCC CGG Ala 1410 * ACC TGG	CCC GGG Pro CTG GAC	* ATC TAG Ile * CCC GGG	130 GAG CTC Glu 14 CCA GGT	50 * AAA TTT Lys 20 * TCC AGG	* ACC TGG Thr * CGG GCC	1: ATC TAG Ile 1 GAT CTA	370 * TCC AGG Ser 430 * GAG CTC	AAA TTT Lys CTG GAC	* GCC CGG Ala * ACC TGG	1380 * AAA TTT Lys> 1440 * AAG TTC
TGC ACG Cys GGG CCC Gly	* AAG TTC Lys * CAG GTC Gln	13: GTC CAG Val 13: CCC GGG Pro	30 * TCC AGG Ser 90 * CGA GCT Arg	* AAC TIG Asn * GAA CTT Glu	13 AAA TTT Lys 14 CCA GGT Pro	GCC CGG Ala 400 * CAG GTC Gln	CTC GAG Leu GTG CAC Val	* GGT Pro * TAC ATG Tyr	GCC CGG Ala 1410 * ACC TGG Thr	CCC GGG Pro CTG GAC Leu	* ATC TAG Ile * CCC GGG Pro	130 GAG CTC Glu 14 CCA GGT Pro	50 * AAA TTT Lys 20 * TCC AGG Ser	* ACC TGG Thr * CGG GCC Arg	1 ATC TAG Ile 1 GAT CTA Asp	370 * TCC AGG Ser 430 * GAG CTC Glu	AAA TTT Lys CTG GAC Leu	* GCC CGG Ala * ACC TGG Thr	AAA TTT Lys> 1440 * AAG TTC Lys>
TGC ACG Cys GGG CCC Gly	* TTC Lys * CAG GTC Gln	13: GTC CAG Val 13: CCC GGG Pro 14	30 * TCC AGG Ser 90 * CGA GCT Arg	* AAC TIG Asn * GAA CTT Glu	1: AAA TTT Lys 1 CCA GGT Pro	GCC CGG Ala 400 * CAG GTC Gln 460	CTC GAG Leu GTG CAC Val	* GGT Pro * TAC ATG Tyr	GCC CGG Ala 1410 * ACC TGG Thr 1470	CCC GGG Pro CTG GAC Leu	* TAG Ile * CCC GGG Pro	130 GAG CTC Glu 142 CCA GGT Pro 14	50 * AAA TTT Lys 20 * TCC AGG Ser 80	* ACC TGG Thr * CGG GCC Arg	1 ATC TAG Ile 1 GAT CTA Asp	370 * TCC AGG Ser 430 * GAG CTC Glu 490	AAA TTT Lys CTG GAC Leu	* GCC CGG Ala * ACC TGG Thr	1380 * AAA TTT Lys> 1440 * AAG TTC Lys> 1500
TGC ACG Cys GGC GCC Gly	* TTC Lys * CAG GTC Gln *	13: GTC CAG Val 13: CCC GGG Pro 14	30 * TCC AGG Ser 90 * CGA GCT Arg 50 *	* AAC TIG Asn * GAA CTT Glu	1: AAA TTT Lys 1 CCA GGT Pro 1	GCC CGG Ala 400 * CAG GTC Gln 460	CTC GAG Leu GTG CAC Val	* GGT Pro * TAC ATG Tyr	1350 * GCC CGG Ala 1410 * ACC TGG Thr 1470 *	CCC GGG Pro CTG GAC Leu	* TAG Ile * CCC GGG Pro	130 GAG CTC Glu 14 CCA GGT Pro 14	50 * AAA TTT Lys 20 * TCC AGG Ser 80 *	* ACC TGG Thr * CGG GCC Arg	1 ATC TAG Ile 1 GAT CTA Asp	370 * TCC AGG Ser 430 * GAG CTC Glu 490 *	AAA TTT Lys CTG GAC Leu	* GCC CGG Ala * ACC TGG Thr *	1380 * AAA TTT Lys> 1440 * AAG TTC Lys> 1500 *
TGC ACG Cys GGG CCC Gly AAC	* AAG TTC Lys * CAG GTC GIn * CAG	13: GTC CAG Val 13: CCC GGG Pro 14 GTC	30 * TCC AGG Ser 90 * CGA GCT Arg 50 *	* AAC TIG Asn * GAA CTT Glu * CTG	1: AAA TTT Lys 1. CCA GGT Pro 1. ACC	GCC CGG Ala 400 * CAG GTC Gln 460 * TGC	CTC GAG Leu GTG CAC Val	* GGT Pro * TAC ATG Tyr * GTC	GCC CGG Ala 1410 * ACC TGG Thr 1470 * AAA	CCC GGG Pro CTG GAC Leu	* ATC TAG Ile * CCC GGG Pro * TTC	130 GAG CTC Glu 14 CCA GGT Pro 14 TAT	50 * AAA TTT Lys 20 * TCC AGG Ser 80 *	* ACC TGG Thr * CGG GCC Arg	1 ATC TAG Ile 1 GAT CTA Asp 1 GAC	370 * TCC AGG Ser 430 * GAG CTC Glu 490 *	AAA TTT Lys CTG GAC Leu	* GCC CGG Ala * ACC TGG Thr * GTG	1380 * AAA TTT Lys> 1440 * AAG TTC Lys> 1500 *
TGC ACG Cys GGG CCC Gly AAC TTG	* TTC Lys * CAG GTC Gln * CAG GTC	13: GTC CAG Val 13: CCC GGG Pro 14 GTC CAG	30 * TCC AGG Ser 90 * CGA GCT Arg 50 * AGC TCG	* AAC TIG Asn * GAA CTT Glu * CTG GAC	13 AAA TTT Lys 14 CCA GGT Pro 14 ACC TGG	40 GCC CGG Ala 400 * CAG GTC GIn 460 * TGC ACG	CTC GAG Leu GTG CAC Val	* GGT Pro * TAC ATG Tyr * GTC CAG	I350 * GCC CGG Ala I410 * ACC TGG Thr 1470 * AAA TTT	CCC GGG Pro CTG GAC Leu GGC CCG	* ATC TAG Ile * CCC GGG Pro * TTC AAG	130 GAG CTC Glu 14 CCA GGT Pro 14 TAT	AAA TTT Lys 20 * TCC AGG Ser 80 *	* ACC TGG Thr * CGG GCC Arg * * AGC TCG	1 ATC TAG Ile 1 GAT CTA Asp 1 GAC CTG	370 * TCC AGG Ser 430 * GAG CTC Glu 490 * ATC TAG	AAA TTT Lys CTG GAC Leu GCC CGG	* GCC CGG Ala * ACC TGG Thr * GTG CAC	AAA TTT Lys> 1440 * AAG TTC Lys> 1500 * GAG CTC
TGC ACG Cys GGG CCC Gly AAC TTG Asn	* AAG TTC Lys * CAG GTC Gln * CAG GTC Gln	13: GTC CAG Val 13: GCC GGG Pro 14 GTC CAG Val	30 * TCC AGG Ser 90 * CGA GCT Arg 50 * AGC TCG Ser	* AAC TIG Asn * GAA CIT Glu * CIG GAC Leu	1: AAA TTT Lys 1. CCA GGT Pro 1. ACC TGG Thr	40 GCC CGG Ala 400 * CAG GTC GIn 460 * TGC ACG Cys	CTC GAG Leu GTG CAC Val CTG GAC Leu	* GGT Pro * TAC ATG Tyr * GTC CAG Val	1350 * GCC CGG Ala 1410 * ACC TGG Thr 1470 * AAA TTT Lys	CCC GGG Pro CTG GAC Leu GGC GGC Gly	* ATC TAG Ile * CCC GGG Pro * TTC AAG	130 GAG CTC Glu 14 CCA GGT Pro 14 TAT ATA TATA	50 * AAA TTT Lys 20 * TCC AGG Ser 80 * CCC GGG Pro	* ACC TGG Thr * CGG GCC Arg * AGC TCG Ser	11 ATC TAG Ile 1 GAT CTA Asp 1 GAC CTG Asp	370 * TCC AGG Ser 430 * GAG GAG CTC Glu 490 * ATCC TAG 90 *	AAA TTT Lys CTG GAC Leu GCC CGG Ala	* GCC CGG Ala * ACC TGG Thr GTG CAC Val	1380 * AAA TTT Lys> 1440 * AAG TTC Lys> 1500 * GAG CTC Glu>
TGC ACG Cys GGG Gly AAC TTG Asn	* AAG TTC Lys CAG GTC GIn * CAG GTC GIn	13: GTC CAG Val 13: CCC GGG Pro 14 GTC CAG Val	30 * TCC AGG Ser 90 * CGA GCT Arg 50 * AGC TCG Ser 10	* AAC TTG Asn * GAA CTT Glu * CTG GAC Leu	1: AAA TTTT Lys 1. CCA GGT Pro 1. ACC TGG Thr 1	GCC CGG Ala 400 * CAG GTC Gln 460 * TGC Cys 520	CTC GAG Leu GTG CAC Val CTG GAC Leu	* CCA GGT Pro * TAC ATG Tyr * GTC CAG Val	1350 * GCC CGG Ala 1410 * ACC TGG Thr 1470 * AAA TTT Lys 1530	CCC GGG Pro CTG GAC Leu GGC Gly	* ATC TAG Ile * CCC GGG Pro * TTC AAG Phe	130 GAG CTC Glu 14 CCA GGT Pro 14 TAT ATA Tyr 15	50 * AAA TTT Lys 20 * TCC AGG Ser 80 * CCC GGG Pro 40	* ACC TGG Thr * CGG GCC Arg * AcC SCG SCC SCC SCC	1: ATC TAG Ile 1 GAT CTA Asp 1 GAC CTG Asp	370 * TCC AGG Ser 430 * GAG GAG CTC Glu 490 * ATC 550	AAA TTT Lys CTG GAC Leu GCC CGG Ala	* GCC CGG Ala * ACC TGG Thr * GTG CAC Val	1380 * AAA TTT Lys> 1440 * AAG TTC Lys> 1500 * GAG CTC Glu> 1560
TGC ACG Cys GGG Gly AAC TTG Asn	* AAG TTC Lys CAG GTC Gln * CAG GTC Gln *	133 GTC CAG Val 133 CCC GGG Pro 14 GTC CAG Val 15	30 * TCC AGG Ser 90 * CGA GCT Arg 50 * AGC TCG Ser 10 *	* AAC TTG Asn * GAA CTT Glu * CTG GAC Leu	13 AAA TTT Lys 1 CCA GGT Pro 1. ACC TGG Thr 1	GCC CGG Ala 400 * CAG GTC Gln 460 * TGC Cys 520 *	CTC GAG Leu GTG CAC Val CTG GAC Leu	* CCA GGT Pro * TAC ATG Tyr * GTC CAG Val *	1350 * GCC CGG Ala 1410 * ACC TGG Thr 1470 * AAA TTT Lys 1530 *	CCC GGG Pro CTG GAC Leu GGC Gly	* ATC TAG Ile * CCC GGG Pro * TTC AAG Phe	130 GAG CTC Glu 14: CCA GGT Pro 14 TAT ATA Tyr 15	50 * AAA TTT Lys 20 * TCC AGG Ser 80 * CCC GGG GGG Pro 40 *	* ACC TGG Thr * * CGG GCC Arg * ACC * * * * * * * * * * * * * * * * *	1 ATC TAG Ile 1 GAT CTA Asp 1 GAC CTG Asp 1	370 * TCC AGG Ser 430 * GAG CTC Glu 490 * ATC 550 *	AAA TTT Lys CTG GAC Leu GCC CGG Ala	* GCC CGG Ala * ACC TGG Thr & GTG CAC Val	1380 * AAA TTT Lys> 1440 * AAG TTC Lys> 1500 * GAG CTC Glu> 1560 *
TGC ACG Cys GGG Gly AAC TTG Asn	* AAG TTC Lys CAG GTC Gln * CAG GTC Gln * GAG	13: GTC CAG Val 13: CCC GGG Pro 14 GTC CAG Val 15 AGC	30 * TCC AGG Ser 90 * CGA GCT Arg 50 * AGC TCG Ser 10 *	* AAC TTG Asn * GAA CTT Glu * CTG GAC Leu *	13 AAA TTT Lys 1 CCA GGT Pro 1. ACC TGG Thr 1 CAG	GCC CGG Ala 400 * CAG GTC Gln * TGC Cys 520 *	CTC GAG Leu GTG CAC Val CTG GAC Leu	* GGT Pro * TAC ATG Tyr * GTC CAG Val *	1350 * GCC CGG Ala 1410 * ACC TGG Thr 1470 * 1470 * 1530 *	CCC GGG Pro CTG GAC Leu GGC Gly TAC	* ATC TAG Ile * CCC GGG Pro * TTC AAG Phe *	130 GAG CTC Glu 142 CCA GGT Pro 14 TAT ATA Tyr 15 S ACC	50 * AAA TTT Lys 20 * TCC AGG Ser 80 * CCC GGG Pro 40 *	* ACC TGG Thr * * CGG * GCC * Arg * * AGC * * * * * * * * * * * * * * * * * * *	1 ATC TAG Ile 1 GAT CTA Asp 1 GAC CTG Asp 1 GAC	370 * TCC AGG Ser 430 * GAG CTC GAG CTC Glu 490 * ATCC Ser * 430 * GAG GAG GAG GAG CTCC * GAG GIU * * * * * * * * * * * * *	AAA TTT Lys CTG GAC Leu GCC CGG Ala	* GCC CGG Ala * ACC TGG Thr CGC CAC Val *	1380 * AAA TTT Lys> 1440 * AAG TTC Lys> 1500 * GAG CTC Glu> 1560 * TCC
TGC ACG Cys GGG CCC Gly AAC TTG Asn	* AAG TTC Lys CAG GTC Gln * CAG GTC Gln * GAG CTC	13: GTC CAG Val 13: CCC GGG Pro 14 GTC CAG Val 15 AGC TCG	30 * TCC AGG Ser 90 * CGA GCT Arg 50 * AGC TCG Ser 10 * AAT	* AAC TTG Asn * GAA CTT Glu * CTG GAC Leu *	13 AAA TTT Lys 1 CCA GGT Pro 1 ACC TGG Thr 1 CAG GTC	GCC CGG Ala 400 * CAG GTC Gln * TGC CAG GTC CJS * 520 * CSS *	CTC GAG Leu GTG CAC Val CTG GAC Leu GAG CTC	* GGT Pro * TAC ATG Tyr * GTC CAG Val * AAC	1350 * GCC CGG Ala 1410 * ACC TGG Thr 1470 * 1470 * 1530 * 1530 *	CTC GGG Pro CTG GAC Leu GGC Gly TAC	* ATC TAG Ile * CCC GGG Pro * TTC AAG Phe * AAG	130 GAG CTC Glu 14: CCA GGT Pro 14 TAT ATA Tyr 15 : ACC : TGG	50 * AAA TTT Lys 20 * TCC AGG Ser 80 * CCC GGG Pro 40 * 20 * CCC 5 * TGC *	* ACC TGG Thr * * GGG * GGC * Arg * * AGC * * AGC * * * * * * * * * * * * * * * * * * *	1: ATC TAG Ile 1 GAT CTA Asp 1 GAC CTG Asp 1 GAC CTG GGC	370 * TCC AGG Ser 430 * GAG GAG GAG GIU * 490 * 490 * 550 * GTC GTC GTC GTC GTC GTC GTC GTC	AAA TTT Lys CTG GAC Leu GCC CGG Ala	* GCC CGG Ala * ACC TGG Thr GTG CAC Val * ; GAC	1380 * AAA TTT Lys> 1440 * AAG TTC Lys> 1500 * GAG CTC Glu> 1560 * TCC AGG
TGC ACG Cys GGG Gly AAC TTG Asn TGG ACC Trp	* AAG TTC Lys CAG GTC GIn * CAG GTC GIn * GAG CTC GIu	13: GTC CAG Val 13: CCC GGG Pro 14 GTC CAG Val 15 AGC TCG Ser	30 * TCC AGG Ser 90 * CGA GCT Arg 50 * AGC TCG Ser 10 * AAT AAT	* AAC TTG Asn * GAA CTT Glu * CTG GAC Leu * * GAC CTG GAC Leu	13 AAA TTTT Lys GGT Pro 1 ACC TGG Thr 1 CAG GTC GIn	340 GCC CGG Ala 400 * CAG GTC Gln 460 * TGC ACG Cys 520 * CCG Pro	CTC GAG Leu GTG CAC Val CTG GAC Leu GAG CTC	* GGT Pro * TAC ATG Tyr * GTC CAG Val * AAC	1350 * GCC CGG Ala 1410 * ACC TGG Thr 1470 * AAA TTT Lys 1530 * * AAC * * * * * * * * * * * * *	CTG GGC Pro CTG GAC Leu GGC Gly TAC ATG Tyr	* ATC TAG Ile CCC GGG Pro * TTC AAG Phe * AAG CCC · Lys	130 GAG CTC Glu 14: CCA GGT Pro 14 TAT ATA Tyr 15 ACC TGG	50 * AAA TTT Lys 20 * TCC AGG Ser 80 * CCC 633 Pro 40 * ACG TCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCC 5 CCCC 5 CCC 5 CCCC 5 CCC 5 CCCC 5 CCCC 5 CCC 5 CCCC 5 CCCC 5 CCCC 5 CCCC 5 CCCC 5 CCCC 5 CCCC 5 CCCC 5 CCCC 5 CCCC 5 CCCC 5 CCCC 5 CCCC 5 CCCCC 5 CCCCC 5 CCCCC 5 CCCCCC 5 CCCCCCCC	* ACC TGG Thr * CGG GCC Arg * CGG * Arg * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * CGG * C CGG * C C C C	1: ATC TAG Ile 1 GAT CTA Asp 1 GAC CTG Asp 1 GAC CCC GCC CCC GCC CCC	370 * TCC AGG Ser 430 * 430 * GAG GAG GAG CTC Glu 490 * 2550 * CTC CTC GLU * 430 * * CTC CTC CTC CTC CTC CTC CT	AAA TTT Lys CTG GAC Leu GCC CGG Ala CTG CGG CGG CGG	* GCC CGG Ala * ACC TGG Thr GTG CAC Val * GAC CAC Val	1380 * AAA TTT Lys> 1440 * AAG TTC Lys> 1500 * GAG CTC Glu> 1560 * TCC AGG Ser>

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

## Fig.10D.

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

16901700***CTCTCCCTGTCTCCGGGTAAATGAGAGAGGGACAGAGGGSerAcaGGCLeuSerLeuSerProGlyLys







## Fig.13A.

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TAC	Val	Sor	AIG	ACC	Aen	100 Thr	GIV	Val	Leu	Leu	Cvs	Ala	Leu	Leu	Ser	Cvs	Leu	Leu	Leu>
Met	vai	Jer	TÄT	пр	nap		U1J	vui	200	200	010								
		7	0			80			90			10	00		1	10			120
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ACA	gga	TCT	AGT	TCA	GGT	TCA	AAA	TTA	<b>AAA</b>	GAT	CCT	GAA	CTG	AGT	TTA	AAA	GGC	ACC	CAG
TGT	CCT	AGA	TCA	AGT	CCA	AGT	TTT	AAT	TTT	CTA	GGA	CTT	GAC	Sor	AAT Leu	ING	Glv	Thr	Glus
Inr	GIY	Ser	Ser	Ser	GIY	ser	ъуз	rea	гуз	Asp	PIU	Giù	Deu	Jer	DCU	<b>DJS</b>	011		Gim
		13	30		1	40			150			1	60		:	170			180
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CAC	ATC	ATG	CAA	GCA	GGC	CAG	ACA	CTG	CAT	CTC	CAA	TGC	AGG	GGG	GAA	GCA	GCC	CAT	ААА
GIG	TAG	TAC	GTT	CGT	CCG	GTC	TGT	GAC	GTA	GAG	GTT	ACG	TCC	ccc	CIT	CGT	CGG	GTA	TTT
His	Ile	Met	Gln	Ala	Gly	Gln	Thr	Leu	His	Leu	Gin	Cys	Arg	GIÀ	GIU	Ala	ALA	HIS	rÀ2>
		19	20			200			210			2	20			230			240
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TGG	TCT	TTG	CCT	GAA	ATG	GTG	AGT	AAG	GAA	AGC	GAA	AGG	CIG	AGC	ATA	ACT	AAA	TCT	GCC
ACC	AGA	AAC	GGA	CTT	TAC	CAC	TCA	TTC	CTT	TCG	CTT	TCC	GAC	TCG	TAT	TGA	TTT	AGA	CGG
Trp	Ser	Leu	Pro	Glu	Met	Val	Ser	Lys	Gļu	Ser	Glu	Arg	Leu	Ser	Ile	Thr	Lys	Ser	Ala>
		~	- •						270			2	80			290			300
	*	2	50 *	*		260		*	270		*	2	*	*		*		*	*
TGT	GGA	AGA	AAT	GGC	ААА	САА	TTC	TGC	AGT	ACT	TTA	ACC	TIG	AAC	ACA	GCT		GCA	AAC
ACA	CCT	TCT	TTA	CCG	TTT	GTI	AAG	ACG	TCA	TGA	AAT	TGG	, AAC	TTG	TGI	CG2	GTI	CGI	TTG
Cys	Gly	Arg	Asn	Gly	Lys	Gln	Phe	Cys	Ser	Thr	Leu	Thr	: Leu	Asn	Thr	Ala	ı Glr	ı Ala	ı Asn>
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GTG	TGA		AAG	ATG	TCG	ACG	TTT	ATA	GAI	CGA	CAI	GGZ	TGA	AGI	TTC	TTC	: TTC	CT	r TGT
His	Thr	Gly	Phe	тут	Ser	Cys	: Lys	Тут	Leu	Ala	Val	Pro	o Thr	Ser	Lys	; Lys	5 Lys	s Glu	1 Thr>
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	+	3	70			380		*	390	) T	*	4	*			410		*	*
GAA	יייעריי. עריידעריי	י הרא	- 	דע די	מידא י	יייזיים. יייזיים	וית ח	AGT	GAT		GG1	r agi	A CCI	TTC	GT	A GA	G AT	G TA	C AGT
CTI	AGA		TAC	S ATA	TAT	- AA	A TAZ	TC	CT7	A TGI	r ccz	A TC	r GG2	A A A	G CA	r cr	C TA	C AT	g tca
Glu	ı Ser	Ala	a Ile	э Тут	Ile	e Phe	e Ile	e Sei	As	o Thi	Gly	Ar	g Pro	o Phe	e Va	l Gl	u Me	t Ty	r Ser≻
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GN	* \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \		י האזי	יייתי א דייתי א	מיתא י		י אחמי	ריי אריי	גבוסיו		A AGO	G GA	G CT	GT	. AT	r cc	C TG	c co	G GTT
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Glu	1 Ile	e Pro	Glu	ı Ile	e Ile	e His	s Met	Thu	c Gli	ı Gly	y Arg	g Gl	u Lei	ı Val	1 11	e Pr	o Cy	s Ar	g Val>
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Th	r Se	r Pr	o As	n Il	e Th	r Va	1 Th	r Le	u Ly	s Ly	s Ph	e Pr	o Le	u As	p Th	r Le	u Il	e Pr	:o Asp>

### Fig.13B

640 650 660 630 620 610 * * * + * * * * GAA ATA GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT CTT TAT CCC GAA GAC TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr>

730740750760770780***************************************************************************************************************************************************************************************************<td

790800810820830840*********AGA GTT CAA ATG ACC TGG AGT TAC CCT GAT GAA ATT GAC CAA AGC AAT TCC CAT GCC AACTCT CAA GTT TAC TGG ACC TCA ATG GGA CTA CTT TAA CTG GTT TCG TTA AGG GTA CGG TTGArg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Ile Asp Gln Ser Asn Ser His Ala Asn>

910 920 930 940 950 960 * TGT CGT GTA AGG AGT GGA CCA TCA TTC AAA TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT ACA GCA CAT TCC TCA CCT GGT AGT AAG TTT AGA CAA TTG TGG AGT CAC GTA TAT ATA CTA Cys Arg Val Arg Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr Asp>

970980990100010101020**********AAA GCA GGC CCG GGC CGG CGC GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCATTT CGT CCG GGC CCG CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT ACG GGT GGC ACG GGTLys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro>

1030 1040 1050 1060 1070 1080 CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT TTT GGG TTC CTG TGG Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr>

## Fig.13C.

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

1550 1540 * * 1520 * 1560 1530 1510 * * * * TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu>

1590 1570 1580 1600 1610 1620 1600 * * . * * ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu>

# Fig.13D.

1660 1670 1650 1630 1640 * * * * * × × * * GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG GAG AGG GAC AGA GGC CCA TTT ACT Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>

Fig. 14A. 30 40 50 60 ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu> 90 100 110 120 80 70 * * * * ACA GGA TCT AGT TCC GGA GGT AGA CCT TTC GTA GAG ATG TAC AGT GAA ATC CCC GAA ATT TGT CCT AGA TCA AGG CCT CCA TCT GGA AAG CAT CTC TAC ATG TCA CTT TAG GGG CTT TAA Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile> 160 170 180 150 130 140 * * * * ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT ACG TCA CCT AAC ATC TAT GIG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA TGC AGT GGA TIG TAG Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile> 230 240 200 210 220 190 * * * * * ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT GGA AAA CGC ATA ATC TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA CCT TTT GCG TAT TAG Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile> 290 300 270 280 250 260 * 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> 350 360 330 340 320 310 ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT CTC ACA CAT CGA CAA TGG ACA CTT CGT TGT 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 Gln> 420 390 400 410 370 380 * * * - -ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC AAA TTA CTT AGA GGC TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG TTT AAT GAA TCT CCG Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly> 470 480 430 440 450 460 * * * * * 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> 530 540 500 510 520 490 * TOG AGT TAC CCT GAT GAA ATT GAC CAA AGC AAT TCC CAT GCC AAC ATA TTC TAC AGT GTT ACC TCA ATG GGA CTA CTT TAA CTG GTT TCG TTA AGG GTA CGG TTG TAT AAG ATG TCA CAA Trp Ser Tyr Pro Asp Glu Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val>

### Fia.14B.

580 590 550 560 570 600 * * * * CTT ACT ATT GAC AAA ATG CAG AAC AAA GAC AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT GAA TGA TAA CTG TTT TAC GTC TTG TTT CTG TTT CCT GAA ATA TGA ACA GCA CAT TCC TCA Leu Thr Ile Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser> 640 650 660 610 620 630 * * * * * * * GGA CCA TCA TTC ANA TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC CCT GGT AGT AAG TTT AGA CAA TTG TGG AGT CAC GTA TAT ATA CTA TTT CGT CCG GGC CCG Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly> 670 680 690 700 710 720 * * * * GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu> 770 760 780 730 740 750 * * * ٠ * * * * GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg> 840 820 830 790 800 810 * * * * * * * * ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe> 900 880 890 850 860 870 * * * * AAC TEG TAC ETE GAC EEC ETE GAG ETE CAT AAT ECC AAG ACA AAG CCE CEE 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 Ala Lys Thr Lys Pro Arg Glu Glu Gln> 960 930 940 950 910 920 * * * * * 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 Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn> 1010 1020 1000 990 970 980 * * * * * * *

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>

1060 1070 1050 1080 1040 1030 * 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 Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg>

## Fig.14C.

1120 1130 1140 1090 1100 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>

1190 1200 1180 1150 1160 1170 * * * * * * * * 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>

1240 1250 1260 1210 1220 1230 * * * * * * * CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser>

1310 1320 1300 1270 1280 1290 * * * * ٠ * * * * * 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 TGC GTC TTC TCG GAG AGG GAC AGA GGC CCA TTT ACT Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>

## Fig.15A.

40 50 60 10 20 30 ATG GTC AGC TAC TOG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu> 110 120 70 80 90 100 ACA GGA TCT AGT TCC GGA GGT AGA CCT TTC GTA GAG ATG TAC AGT GAA ATC CCC GAA ATT TGT CCT AGA TCA AGG CCT CCA TCT GGA AAG CAT CTC TAC ATG TCA CTT TAG GGG CTT TAA Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile> 170 160 180 130 140 150 * * * * * * * ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT ACG TCA CCT AAC ATC TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA TGC AGT GGA TTG TAG Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile> 220 230 240 190 200 210 * * * * * ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT GGA AAA CGC ATA ATC TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA CCT TTT GCG TAT TAG Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile> 270 280 290 300 250 260 * * 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> 330 340 350 360 310 320 * * * * * ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT CTC ACA CAT CGA CAA TGG ACA CTT CGT TGT 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 Gln> 420 390 400 410 370 380 * * ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC AAA TTA CTT AGA GGC TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG TTT AAT GAA TCT CCG Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly> 460 470 480 430 440 450 * * * * 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> 540 510 520 530 490 500 * TGG AGT TAC CCT GAT GAA AAA AAT AAG AGA GCT TCC GTA AGG CGA CGA ATT GAC CAA AGC

TGG AGT TAC CCT GAT GAA AAA AAT AAG AGA GCT TCC GTA AGG CGA CGA ATT GAC CAA AGC ACC TCA ATG GGA CTA CTT TTT TTA TTC TCT CGA AGG CAT TCC GCT GCT TAA CTG GTT TCG Trp Ser Tyr Pro Asp Glu Lys Asn Lys Arg Ala Ser Val Arg Arg Arg Ile Asp Gln Ser> Fig.15B.

590 600 570 580 560 550 * * * * * AAT TCC CAT GCC AAC ATA TTC TAC AGT GTT CTT ACT ATT GAC AAA ATG CAG AAC AAA GAC TTA AGG GTA CGG TTG TAT AAG ATG TCA CAA GAA TGA TAA CTG TTT TAC GTC TTG TTT CTG Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys Asp> 650 660 620 630 640 610 * * * + * * AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT GGA CCA TCA TTC AAA TCT GTT AAC ACC TCA TTT CCT GAA ATA TGA ACA GCA CAT TCC TCA CCT GGT AGT AAG TTT AGA CAA TTG TGG AGT Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser> 700 710 720 690 670 680 * * * * * GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA CAC GTA TAT ATA CTA TTT CGT CCG GGC CCG CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr> 770 760 7B0 750 730 740 * ٠ . TEC CCA CCE TEC CCA ECA CCT GAA CTC CTE GEE GEA COE TCA ETC TTC CTC TTC CCC CCA ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro> 820 830 840 810 790 800 * * * AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG CAC CAC CTG Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp> 900 890 880 850 860 870 * * * • . * GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His> 960 930 940 950 910 920 * * * * * * AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val> 1020 970 980 990 1000 1010 * CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn> 1070 1080 1040 1050 1060 1030 * * * AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA

TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu>

## Fig.15C.

1100 1110 * * * * * 1120 1130 * * * 1090 1140 * * * 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> 1150 1160 1170 1180 1190 1200 * * * * * * * * * ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG TGG ACG GAC CAG TTT CCG AAG ATA GGG TCG 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> 1240 1210 1220 * 1260 1230 1250 * * * * * * * CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC GTC GGC CTC TTG TTG ATG TTC TOG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe> 1270 1280 1290 1300 1310 * * * * * * * * * 1320 * CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC GAG ATG TOG TTC GAG TGG CAC CYG TTC TOG TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys> 1340 1350 1360 1370 1380 * * * * * * * * * * 1330 * * TCC 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 TTT ACT Gly Lys ***>

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# Fig.16A.

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Met	Val	Ser	Tyr	Trp	Asp	Thr	Gly	Val	Leu	Leu	Cys	Ala	Leu	Leu	Ser	Cys	Leu	Leu	Leu>
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TGT	CUT	AGA	For	AGT	CUA	Cor	TAT	Lou	Tare	AcD.	Bro	Glu	Leu	Ser	Leu	LVG	GIV	Thr	Gln>
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CAC	ATC	ATG	CAA	GCA	GGC	CAG	ACA	CTG	CAT	CTC	CAA	TGC	AGG	GGG	GAA	GCA	GCC	CAT	ААА
GTG	TAG	TAC	GTT	CGT	CCG	GTC	TGT	GAC	GTÁ	GAG	GTT	ACG	TCC	CCC	CTT	$\mathbf{CGT}$	CGG	GTA	TTT
His	Ile	Met	Gln	Ala	Gly	Gln	Thr	Leu	His	Leu	Gln	Cys	Arg	Gly	Glu	Ala	Ala	His	Lys>
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TGG	TCT	TIG	CCT	GAA	ATG	GIG	AGT	AAG	GAA	AGC	GAA	AGG	CIG	AGC	ATA	ACT	AAA	TCT	GCC
ACC	AGA	AAC	GGA	CTT	TAC	CAC	TCA	TIC	CIT	TCG	CPP	TCC	GAC	106	TAT	AUT: A	-1-1-1 -	AejA	
IID	Ser	Leu	Pro	GIU	met	vai	Ser	Lys	Gru	ser	Gru	Arg	Leu	ser	TTE	THE	Lys	Ser	AId>
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ACA	CCT	TCT	TTA	CCG	TTT	GTT	AAG	ACG	TCA	TGA	AAT	TGG	AAC	TTG	TGT	CGA	GTI	CGT	TTG
Cys	Gly	Arg	Asn	Gly	Lys	Gln	Phe	Cys	Ser	Thr	Leu	Thr	Leu	Asn	Thr	Ala	Gln	Ala	Asn>
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CAC	ACT	GGC	TTC	: TAC	AGC	TGC	AAA	TAT	CTA	GCT	GTA	CCI	ACT	TCA	AAG	AAG		GAA	ACA
GIG	TGA	CCG	AAG	ATG	TCG	ACG	TTT	ATA	GAT	CGA	CAT	GGA	TGA	AGI	TIC	TTC	TIC	CTI	TGT
His	Thr	Gly	Phe	e Tyr	Ser	Cys	Lys	Тут	Leu	ALA	vai	Pro	o mr	· ser	Lys	Lys	: LYS	GIU	Thr>
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CTI	AGA	CGI	TAC	G ATZ	A TAT	אאא י	TAA	TCA	CT7	TGI	CCZ	TCI	GGI	. AAC	CAT	r CTC	. TA	: ATC	TCA
Glu	Ser	Ala	Ile	э Тут	: Ile	Phe	Ile	e Ser	Asp	'Thr	Gly	Arc	y Pro	) Phe	val	l Glu	ı Met	: Tyr	Ser>
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CTI	TAC	GGC	G CT	F TAZ	A TAT	r GTG	S TAC	TG2	A CT		r TCC	CTO	GA	G CAC	G TAL	A GGO	G AC	G GCC	
GIU	1 Ile	e Pro	o Glu	u Ile	e Ile	e His	s Met	: Th	GI	1 GI	Y Arg	1 GTI	i rei	ı va.	ь <u>,11</u>	e pro	о су	s ar	y var>
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TGO	AG	r GG	ATT	G TA	GTG		A TG	A AA	г тт	r TT	CAA	A GG	T GA	A CT	GТG	<b>A</b> A A	C TA	GGG	A CTA
Th	Se	r Pre	o As	n Il	e Th	r Val	1 Th	r Le	u Ly:	s Ly	s Ph	e Pr	o Le	u As	p Th	r Le	u Il	e Pr	o Asp>

### Fig.16B.

550560570580590600********GGA AAA CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAACCT TTT GCG TAT TAG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTTGly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys>

640 650 660 610 620 630 * * * * * * * GAA ATA GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT CTT TAT CCC GAA GAC TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr>

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 AAA TTA CTT AGA GGC CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC CCC TTG AAC ACG
 TTT AAT GAA TCT CCG GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG GGG AAC TTG TGC
 TTG TGC
 TTG TGC

 Lys Leu Leu Arg Gly His Thr Leu Val
 Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Tr>

790800810820830840********AGA GTT CAA ATG ACC TGG AGT TAC CCT GAT GAA AAA AAT AAGAAC GCT TCC GTA AGG CGATCT CAA GTT TAC TGG ACC TCA ATG GGA CTA CTT TTT TTA TTC TTG CGA AGG CAT TCC GCTArg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys Asn Lys Asn Ala Ser Val Arg Arg>

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 CGA ATT GAC CAA AGC AAT TCC CAT GCC AAC ATA TTC TAC AGT GTT CTT ACT ATT GAC AAA
 GCT TAA CTG GTT TCG TTA AGG GTA CGG TTG TAT AAG ATG TCA CAA GAA TGA TAA CTG TTT

 AGC TAA CTG GTT TCG TAA AGG GTA CGG TTG TAT AAG ATG TCA CAA GAA TGA TGA CTG TTT

 Arg Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys>

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# Fig.16C.

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AAG GAG	AAG	GGG	GGT	TTT	GGG	TTC	CIG	TGG	GAG	TAC	TAG	AGG	GCC	TGG	GGA	CIC	CAG	TGT
Phe Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr>
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TGC GTC	GTG	GTG	GAC	GTG	AGC	CAC	GAA	GAC	CCT	GAG	GTC	AAG	TTC	AAC	TGG	TAC	GTG	GAC
ACG CAC	CAC	CAC	CTG	CAC	TCG	GTG	CTT	CTG	GGA	CTC	CAG	TTC	AAG	TTG	ACC	ATG	CAC	CIG
Cys Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	Val	Asp>
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GGC GTC	GAG	GTG	CAT	AAT	GCC	aag	ACA	AAG	CCG	CGG	GAG	GAG	CAG	TAC	AAC	AGC	ACG	TAC
CCG CAC	CTC	CAC	GTA	TTA	CGG	TTC	TGT	TTC	GGC	GCC	CTC	CTC	GTC	ATG	TIG	TCG	TGC	ATG
Gly Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr>
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CGT GT	GTC	AGC	GTC	CTC	ACC	GTC	CTG	CAC	CAG	GAC	TGG	CTG	ААТ	GGC	AAG	GAG	TAC	AAG
GCA CA	CAG	TCG	CAG	GAG	TGG	CAG	GAC	GTG	GTC	CTG	ACC	GAC	TTA	CCG	TTC	CTC	ATG	TTC
Arg Va	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	· Lys>
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ACG TT		AGG	TTG	TTT	CGG	GAG	GGT	CGG	GGG	TAG	CTC	TTT	TGG	TAG	AGG	TTT	CGG	; TTT
Cys Ly:	s Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys>
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CCC GT	CGGG	GCT	CTT	GGT	GTC	CAC	ATG	TGG	GAC	GGG	GGT	AGG	GCC	CTA	CTC	GAC	TG	3 TTC
Gly Gl	n Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Asp	Glu	Lev	Th	c Lys>
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TTG GT	C CAC	TCG	GAC	TGG	ACG	GAC	CAC	TTI	000	AAG	; ATA	GGG	S TCG	CTC	TAC	GCGC	CAC	C CTC
Asn Gl	n Val	l Ser	Leu	1 Thr	Cys	Lev	val	. Lys	Gly	7 Phe	• Тут	Pro	o Ser	: Asr	o Ile	e Ala	ı Va	l Glu>
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ACC CT	C TCC	5 TTA		GTC	GGC	CTC	TTC	G TTO	S'ATC	TTC	TG	J TGO	GGł	A GG(	G CA	GAQ	CT C	g agg
Trp Gl	u Sei	r Asr	n <b>Gl</b> y	/ Glr	Pro	Glu	i Asi	n Asr	тул	: Lys	s Thi	r Thi	r Pro	o Pro	o Va	l Lei	א ג As	p Ser>
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	÷.	*	,	•	*		*	1	- k	*	_	*		*	*		*	*
GAC GG	c to	C TT	C TR	CTC	: TAC	age	2 AAG	G CTX	C ACC	C GTC	G GAG	C AA	g ago	C AG	G TG	G CA	G CA	.G GGG
CTG CC	GAG	G AAG	G AAG	G GAC	ATC	S TCO	G TT	C GAG	s TG	G CAC	CTX	g TT	c TCC	с то	C AC	C GT	C GT	c ccc
Asp Gl	y Se	r Pho	e Phe	e Lev	тул к	s Se:	r Ly:	s Lei	i Th	r Val	l As	p Ly	s Se:	r Ar	g Tr	p Gl	n Gl	.n Gly>

## Fig.16D.

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

1690 1700 * * * CTC TCC CTG TCT CCG GGT AAA TGA GAG AGG GAC AGA GGC CCA TTT ACT Leu Ser Leu Ser Pro Gly Lys ***>







Fig	.21A.			>EcoRI	_site		
10	20	30	40	50	60	70	80
AGCTTGGGGCTGCP	GGTCGATCGA	TCTAGAGGA	TCGATCCCCC	GGCGAGCTCG	AATTCGCAAC	CACCATGGTC	AGCTAC
CGAACCCGACGI	CCAGCTAGCI	GAGATCICCI	AGCTAGGGG	CUGCIUGAGC	TTAAGCGTTG	M V	S V
						1	4
							>
					>BenET bri	dae	
90	100	110	120	130	140	150	160
GGACACCGGGG	ICCTGCTGTGO	GCGCTGCTCZ	GCTGTCTGC	PICTCACAGGA	TCTAGTTCCC	GAGGTAGACC	TTICGT
CCTGTGGGCCCC	AGGACGACACG	CGCGACGAGI	CGACAGACG/	AGAGTGTCCI	AGATCAAGGO	CICCAICIGG	AAAGCA
V D T G V	LLC	ALL	SCLI	LTG	S S>		
		FLT1 SS_			>_	-	
					S	G>	
						GRI	3.
170	180	190	200	210	220	230	240
			HFLT1 D2				
250	260	270	280	290	300	310	320
TAACATCACTGT	TACTTTAAAAA	AGTTICCAC	TIGACACITI	GATCCCTGAT	GAAAACGCA	TAATCTGGGA	CAGTAGA
ATTGTAGTGACA	ATGAAATTTTT	TCAAAGGTG	AACTGTGAAA	CTAGGGACTA	CTTTIGCGT	ATTAGACCCT	STCATCT
ΝΙΤΥ	TLK	KFPI	LDTL	IPD	GKR	IIWD	S R> 84
			HFLT1 D2				
330	340	350	360	370	380	390	400
AGGGCTICATCA	TATCAAATGC	ACGTACAAA	GAAATAGGGC	TTCTGACCTG	TGAAGCAACA	GTCAATGGGC	
TCCCGAAGTAGT		ידידיבאיי גריביוייו	CTTTATCCCG	ANCACTOCAC	מרייזיייייייייייייייייי	CAGTTACCCG	ATTTGTA
	ATAGTTTACG	rigeniori -					ATTTGTA TAAACAT
KGFI	ATAGTTTACG I S N A	TYK	EIG	LLTC	EAT	VNG	ATTTGTA TAAACAT H L Y
KGFI	ATAGTTTACG I S N A	T Y K	EIG	LLTC	EAT	VNG	ATTTGTA TAAACAT H L Y 1
KGFI	ATAGTITACG' I S N A	T Y K	E I G HFLT1 D2	L L T C	E A T	V N G	ATTTGTA TAAACAT H L Y 1
K G F I	ATAGTITACG' I S N A	TYK	E I G			V N G	ATTTGTA TAAACAT H L Y 1
K G F I 410	ATAGTITACG I S N A 420	430	E I G HFLT1 D2 440	450	Е А Т 460	470	ATTTGTA TAAACAT H L Y 1 480 GAACTAT
K G F I 410 AAGACAAACTAT	ATAGTITACG' I S N A 420 CTCACACATCO	T Y K 430 3ACAAACCAA	E I G HFLT1 D2 440 TACAATCATA	450	E A T 460 TGAGTCCGTC	V N G 470 TCATGGAATT	ATTTGTA TAAACAT H L Y 1 480 GAACTAT CTTGATA
К G F I 410 Алдасаластат Ттстстттсата К Т N Y	ATAGTITACG I S N A 420 CTCACACATCO GAGTGTGTAG L T H I	T Y K 430 GACAAACCAA CIGTIIGGII R Q T N	E I G HFLT1 D2 440 TACAATCATA ATGTTAGTAT T I I	450 GATGTGGTTC CTACACCAAG D>	E A T 460 TGAGTCCGTC ACTCAGGCAG	V N G 470 TCATGGAATT AGTACCTTAA	ATTTGTA TAAACAT H L Y 1 480 GAACTAT CTTGATA
К G F I 410 радасарастат ттстуттурата К Т N Y	ATAGTITACG I S N A 420 CTCACACATCO GAGTGTGTAGG L T H I HFLT1	TYK 430 GACAAACCAA CIGITIGGTI RQTN D2	E I G HFLT1 D2 440 TACAATCATA ATGTTAGTAT T I I	450 GATGTGGTTC CTACACCAAG D> > V V	E A T 460 TGAGTCCGTC ACTCAGGCAG L S P 5	V N G 470 TCATGGAATT AGTACCTTAA	ATTTGIA TAAACAT H L Y 1 480 GAACTAI CTTGATA E L>
К G F I 410 радасарастат ттстуттудата К Т N Y	ATAGTITACG I S N A 420 CTCACACATCO GAGTGTGTAGA L T H I HFLT1	TYK 430 GACAAACCAA CIGITIGGTI RQTN D2	E I G HFLT1 D2 440 TACAATCATA ATGTTAGTAT T I I	450 GATGTGGTTC CTACACCAAG D> > V V	E A T 460 TGAGTCCGTC ACTCAGGCAG L S P S	V N G 470 TCATGGAATT AGTACCTTAA S H G I	ATTTGTA TAAACAT H L Y 480 GAACTAI CTTGATA E L> 137

# Fig.21B.

490	500	510	520	530	540	550	560
CTGTTGGAGAAAA	GCTIGTCTTA	AATIGTACAG	CAAGAACTG	AACTAAATG'	rogggattgaC	ITCAACTGGGA	ATACCCT
GACAACCTCTTTT	CGAACAGAAT	TTAACATGTC	GTTCTTGAC	TIGATITAC	ACCCCTAACTG	AAGTTGACCCT	TATGGGA
SVGEK	LVL	NCTA	ART	ELNV	/GID	FNWE	Y P>
				_			16
			_HFLK1 D	3			
570	500	500	600	610	620	670	640
വസ്വാമവന്മണം സനസ്വാമവന്മണം	300 ACCATAACAA	עבר איזעגעייבאייייייי	000 גרדיריריניבערייניביר	010 יממרוריממממ	020	יייעעענטענטענע אייעענטענטענ	טערט מביצוייודייד
GAAGCTTCGTAG	TCGTATTCTT	GAACATTTG	CTCTGGAT	TTTTGGGTCZ	GACCCTCACT	TACTICITIA	AAAACTC
SSKH	онкк	LVN	RDL	кто	SGSE	MKKH	FLS
	-						1
			_HFLK1 D	3			
				<b>6</b> 0.0			
550	660	670	680	690	700	710	720
ACCITAACIATA NGCDD MDCD MD M	JANGGIGIAAC			STACAUCTGT	GCAGCATCCA		
M I. M T	D C V 1	BGCCICACIO	O G L	V TO C	A A C	ACCUGACIACI	7 V V
TLTL	DGVI	K S D	QЧ	IIC	A A 3 3	, <b>G D</b> M	217
			HELK1 D	2			211
				<u> </u>	<u> </u>	····	
		>Srf_Br	idae_				
		· -					
730	740	750 İ	760	770	780	790	800
2220222022	CTTC ACCOTCC	ATGAAAAGGG		CAAAACTCA	CACATOCCAC	10GTGCCCAGC2	ACCTICA A
		G	; P G> >	איד א	ጥ ቦ ዖ	е <b>с</b> р у	P E>
			-	, K I U	ICF	rtra	24
					FCAC1 (#	v)	
810	820	830	840	850	860	870	880
CCTGGGGGGACC	GTCAGTCTTC	CTCTTCCCCC	CAAAACCCA	AGGACACCC	TCATGATCTCC	CGGACCCCTGA	GGTCAC
GGACCCCCCTGG	CAGICAGAAG	GAGAAGGGGG	GITTIGGGI	TCCIGIGGG	AGTACTAGAGG	GCCIGGGGACT	CCAGIG
LGGF	SVF	Гъъ	ркр	K D T		RTPE	, V 1. 2'
			PCAC1 / A				4
				<u> </u>	·		
890	900	910	920	930	940	950	960
CONCINCING	AUCTORETON	TO TADAAD	O A A T T D D A D	CTTCAACTTC	TACGTGGACGG	CGTGGAGGTGC	ATAATG
	TIGCACTCIGGT	CITCIGGA	CTCCAGTTC	AAGTTGACC	ATGCACCTGCC	GCACCTCCACG	TATTAC
C V V V	DVSH	EDP	EVK	FNW	YVDG	VEV	H N>
	2 . 2			• • • •			297
			FCAC1 (A)				
970	980	990	1000	1010	1020	1030	1040
AAGACAAAGCCG	CGGGAGGAGC	AGTACAACAG	CACGTACCG	TGTGGTCAG	COTCCTCACCG	TCCTGCACCAG	GACTGG
TTCTGTTTCGGO	GCCCTCCTCG?	CATGTIGTO	STGCATGGC.	ACACCAGTC	SCAGGAGTGGC	AGGACGTGGTC	CTGACC
КТКР	REE (	) Y N S	TYR	v v s	VLT	V L Н Q	D W>
							324
			FCAC1 (A)				

# Fig.21C.

FIQ	J.210	<b>*</b> •					
1050	1060	1070	1080	1090	1100	1110	1120
TGAATGGCAAGG	AGTACAAGTG	CAAGGTCTCC	AACAAAGCCC	TCCCAGCCCC	CATCGAGAAA	ACCATCTCCA	AAGCCAA
GACTTACCGTTCC	TCATGTTCAC	GTTCCAGAGG	TIGTITCGGG	AGGGTCGGGG	GTAGCTCTTI	TGGTAGAGGI	MTCGGTT
LNGK	ЕЧКС	. K V S	NKA	LPAP	LEK	TIS	KAK
							3:
·····			FCACI (A)				
				>A	>C_A_allot	ype	
					1		
				>G>T_A	_allotype		
1130	1140	1150	1160	1170	1180	1190	1200
AGGGCAGCCCCGA	GAACCACAGG	TGTACACCCI	GCCCCCATCC	CGGGATGAGC	TGACCAAGA	ACCAGGTCAGO	CTGACCT
ICCCGICGGGGCT	CTIGGIGICC	ACATGTGGGA	CGGGGGTAGG	GCCCTACTCG	ACTGGTTCT	GGTCCAGTCO	GACTGGA
GQPR	EPQ	VYTL	, P P S	RDE	LTKI	IQVS	L T>
							377
			FCACI (A)				
1010	1000	1020	1240	1250	1260	1270	1290
		1230		1230	1200	1270 3707 707 707	
GCCIGGICAAAGG	CHICHAICCC	AGCGACATCG			000000000000000000000000000000000000000	ALANDARY SUPPORT	
	GAAGATAGGG	C D I	A V F M			F N N V	V K m
فكالأك سلحا	FIP	SDI	AVEW	LSN	GQF	ENN	10
			FCAC1 (A)				
				>Ͳ>Ր			
				1			
1290	1300	1310	1320	1330	1340	1350	1360
ACGCCTCCCGTGC	TGGACTCCGA	CGGCTCCTTC	TTCCTCTATA	GCAAGCTCAC	CGTGGACAA	AGCAGGTGG	CAGCAGGG
IGCGGAGGGCACG	ACCTGAGGCT	GCCGAGGAAG	AAGGAGATAT	CGTTCGAGTG	GCACCTGTT	TCGTCCACC	STEGTEEC
TPPV	LDSI	GSF	FLY	SKLT	VDK	SRW	QQG
							4
			FCAC1 (A)				
1370	1380	1390	1400	1410	1420	1430	1440
GAACGTCTTCTCA	TGCTCCGTGA	TGCATGAGGC	TCTGCACAAC	CACTACACGO	AGAAGAGCC	ICTCCCTGTC	ICCGGGTA
CTTGCAGAAGAGT	ACGAGGCACT	ACGTACTCCC	AGACGTGTTG	GTGATGTGCG	TCTTCTCGG	\GAGGGACAG	AGGCCCAT
NVFS	csv	мнед	LHN	н ү т	ок с и	SLS	P G>
							457
			FCΔC1 (A)				
>NotI_site							
1							
1450							
AATGAGCGGCCGC							
ITACTCGCCGGCG	-						
K *>							

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Fig	g.22A	۱.		>EcoR1	[_site		
	-	20	40	50	<b>C</b> 0	70	90
10 AAGCTTGGGCTGCA	20 GGTCGATCGA	30 ACTCTAGAGGA	40 TCGATCCCC	50 GGGCGAGCTCC	GO GAATTCGCAAC	CACCATGGIN	LAGCTAC
TTCGAACCCGACGT	CCAGCTAGCT	GAGATCTCCT	AGCTAGGGG	CCCGCTCGAG	TTAAGCGTTG	GTGGTACCA	FICGATG
						M V 1	SY>
							>
					BenET bri	dae	
						age	
90	100	110	120	130	140	150	160
TGGGACACCGGGGT	CCIGCIGIGC	GCGCTGCTCA	AGCTGTCTGC	TTCTCACAGG!	ATCTAGTTCCG PAGATCAAGGC	GAGGTAGAC( CTCCATCTG)	CTTTCGT GAAAGCA
W D T G V	LLC	A L L	S C L	LLTG	S S>		
	FL/T1	SIGNAL SEC	UENCE		>	•	
					S	G> >	
						GRI	PFV>
							31
170	180	190	200	210	220	230	240
TCTCTACATGTCAC E M Y S	TTTAGGGGC7 E I P I	TTAATATGTG 3 I I H FLJ	STACTGACTT M T E F1 IG DOMA	CCTTCCCTCG	AGCAGTAAGGG LVIP	ACGGCCCAA CRV	TGCAGTG T S> 57 >
				200	200	21.0	320
250 CTAACATCACTGTI	260 ACTITIAAAA2	AGTTTCCACI	280 PTGACACTTI	GATCCCTGAT	GGAAAACGCAI	AATCTGGGA	CAGTAGA
GATTGTAGTGACAA	TGAAATTTT	TCAAAGGTG	ACTGTGAAA	CTAGGGACTA	CCTTTTGCGT	TTAGACCCT	GTCATCT
PNITV	TLK	KFPI	LDTL	IPD	GKRI	IWD	S R> 84
		FL7	ri ig doma	IN 2			>
	240	250	260	270	200	390	400
330 AAGGGCTTCATCAT	340 ATCAAATGCI	350 AACGTACAAA	380 GAAATAGGGC	TTCTGACCTG	TGAAGCAACAG	JJJ0 JTCAATGGGC	ATTIGTA
TTCCCGAAGTAGTA	TAGTTTACG	PIGCATGTTIC	TTTATCCCG	AAGACTGGAC	ACTTCGTTGT	AGTTACCCG	TAAACAT
KGFII	SNA	тук	EIG	LLTC	ЕАТ	VNG	H L Y> 111
		FL7	ri ig doma	IN 2			>
						170	490
410 TANCACARACTERTS	420 ארמרמרמידי	430 מרשמברמי	440 TACAATCATA	450 GATATCCAGC	460 TGTTGCCCAG	470 BAAGTCGCTG	GAGCTGC
ATTCTGTTTGATAG	AGTGTGTAG	TGTTIGGTT	ATGTTAGTAI	CTATAGGTCG	ACAACGGGTCO	TTCAGCGAC	CTCGACG
K T N Y	LTHI	X Q T N	TII	D>			
	FLTI IG D	MAIN 2	· · · · · · · · · · · · · · · · · · ·	 	LLPR	KSL	E L>
							137
				VEG	EKS (FL/14)	TG LOUMIN	· · · · · · · · · · · · · · · · · · ·
Fig.22B.

	CIGO	STCC	TCA	ACTGO	CACC	GTG	TGG	GCTG	AGT	TTAAC	TC	AGG	TGT	CACO	TTT	GAC	CTG	GG/	ACT	AC	CCA
CATCCCCTCTCC	GACC	LAGG	AGT		5TGG m	CAC	:ACC(		TCA F	AATTO FN	aG'.	יית ה	ACA	טעיט ייי	SAA:	TCI T	AC.	:CC!	IGA	viG v	GGT
VGEK	ч	v	r	N C	T	v	YY	n	. ت	r IN	5	G	v	1	£	U	n		0	*	164
				vi	EGFR	3 (	FLT	4) I	GD	OMAIN	¥ 3.										>
								-• -													
570	5	580		59	90		6	00		610	)		6	20		6	530	)			640
GGAAGCAGGCAGA	GCGG	GGI	'AAG'	rggg:	IGCC	CGA	GCG	ACGC	TCC	CAAC	AGA(	ccc	ACA	CAG	AAC I	CT	CCA	AGC.	ATC	CT	GAC
CCTTCGTCCGTCT	CGCC	CCA	TTC	ACCCI	ACGG	GCT	CGC	IGCG	AGG	GTTG	ICIN	GGG	TGT	GTC	PTGA	GAC	GGI -	'CG'	TAG	GA	CTG
GKQAE	R	G	ĸ	W	V P	E	ER	R	S	QC	2 '	T	н	T I	5 L		5	S	Ŧ	L	· T>
				v	FAFR	3 (	T.T.	4) T	Gр	OMAIN	<b>J</b> 3										
						5 (		-, -	-		• •										
650	e	560		6'	70		6	80		690	)		7	00			710	)			720
ATCCACAACGTC	GCC2	AGCA	CGA	CCTG	GCT	CGI	PATG	TGTG	CAA	GGCCZ	AAC.	AAC	:GGC	ATC	CAGC	GA	<b>PP1</b>	ľCG	GGA	AGA	GCA
STAGGTGTTGCAGI	rcgg:	rcgi	GCT	GGAC	CCGA	GCA	ATAC	ACAC	GTT	CCGG?	FTG	TTG	CCG	TAG	STCC	CT.	AA7	łGC	CCI	ГСI	CGT
IHNV	sς	2 E	I D	L	G	s	Y	v c	K	A	N	N	G	I	Q	R	F	R	E	3	S>
																					217
				V	EGFR	3 (	(FLT	4) 1	Gυ	OMAII	N 3										>
720		740		7	50		7	60		770	<b>n</b> .		7	80			790	<b>`</b>			800
		/ -== U ~> > > 7	2000			~~~~	י הההי	້	202			200	, Y291Y2				ייייי דביית	, 	m	<b>.</b>	2000
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						.> D	ĸ	т	н	тс	P	F	, c	P	A	P	I	Ξ	L	L	G> 244
						> D	ĸ	т	н	т с fcd	р С1	- 1		. P	A YPE	P	I	Ξ	L	L	G> 244 >
810	1	820			30	> 	к 	т 40	н	т с fcd( 85)	Р С1 0	- J		: P LOI	A YPE	P	870	Ξ 	L	L	G> 244 > 880
810 SGACCGTCAGTCT	ICCT	820 CTTX		8 CCAA	30 AACC	-> D 	K 8 AGGA	т 40 сасс	н 	т с FCΔ 850 САТСА	P C1 0 ICT	- 1 - 1	P C A AI E CGG7	LOI	A YPE CTG	P	1 87( TC)	2   	L .TGC	L 	G> 244 
810 3GACCGTCAGTCT 2CTGGCAGTCAGAJ	ICCTV	820 CTTX GAA(		8 CCAA GGTT	30 AACC TTGG	-> D 	K 8 AGGA ICCT	T 40 CACO	H 	τ c fcΔ 850 CATGA	P C1 0 ICT AGA	- 1 - 1	e c A AI E CGG7	LOI CCC	A YPE CTG GAC	P	1 87( TC) AG:	E D ACA IGI	L .TGC		G> 244 > 880 IGGT ACCA
810 GACCGTCAGTCT CCTGCCAGTCAGAJ G P S V J	ICCIN AGGA( F L	820 CTTC GAAC F	CCCC CCCC F P	8 CCAA GGTT P	30 AACC TTGG K P	-> D  CAA GTI 	K 8 AGGA ICCT K D	T 40 CACC GTGG T	H CTC GAG L	T C FCΔ 850 CATGA STACTI M	P C1 0 ICT AGA I	- 1 - 1 CCC .GGC S	P C A AI E CGGF FCC7 R	LOI 60 CCC T	A YPE CTGJ GAC ⁴ P	P AGG ICC E	870 7C2 AG1 V	E O ACA IGI T	L .TGC 'ACC		G> 244 
810 GACCGTCAGTCT CCTGGCAGTCAGAJ G P S V J	icctv Agga( F L	820 СТТС БААС F	CCCC SGGG P	8 CCAA GGTT P	30 AACC TTGG K F	> D  CAZ GTI > P	K 8 AGGA ICCT K D	T 40 CACC GTGG T	H CTC GAG L	T C FCΔ B50 CATGA STACT M	P C1 ICT AGA I	- 1 CCC .GGC S	A AI A AI SGGA R	LOI CCC T	A YPE CTGJ GAC P	P AGG ICC	1 87( TC/ AG? V	E D ACA I'GT T	L TGC 'ACC C	L CG.	G> 244 880 IGGT ACCA V V> 27
810 GGACCGTCAGTCT CCTGGCAGTCAGAJ G P S V J	rcctv Aggao F L	820 CTTC GAAC F	cccc secc p	8 CCAA GGTT P	30 AACC TIGG K F	-> D  CA2 SGT1 P F CAC	K 8 AGGA ICCT K D 1 -	T 40 CACC GTGC T A AI	H CTC GAG L	Τ C FCΔ 850 CATGA STACT M TYPE_	P C1 0 ICT AGA I	- 1 - 1 - 2 - 1 - 1 - 1 - 1 - 1 - 1 - 1 - 1 - 1 - 1	e C A AI E CGGZ R R	LOI LOI CCC T	A YPE CTGJ GAC P	P	1 87( TCJ AG: V	E O ACA I'GT T	L TGC VACC C		G> 244 > 880 IGGT ACCA V V> 27 >
810 GGACCGTCAGTCT CCTGGCAGTCAGAJ G P S V 1 	ICCIX AGGA( F L	820 CTTX GAAC F 		8 CCAA GGTT P	30 AACC TTGG K F FC	> D 	K 8 AGGA ICCT K D 1 - 9	T 40 CACC GTGC T A AI	H CCTC GAG L	т с FCΔ 850 САТGА 5ТАСТЛ М :: ГҮРЕ 931	P C1 0 ICT AGA I 0	- I CCCC GGC S	P C A AI E CGGP R R	E P LOI GGG T 40	A YPE CTGJ GAC P	P	1 87( TC) AG! V 95(	E D ACA TGT T	L TGC VACC C		G> 244 > 880 IGGT ACCA V V> 27 > 960
810 GGACCGTCAGTCT CCTGGCAGTCAGAI G P S V 1 890 GGTCGACGTGAGC	ICCTV AGGA( F L	820 CTTC GAAC F 		8 GGTT P 9 TGAG	30 AACC TIGG K F FC	> D 	K 8 AGGA ICCT K D 1 - 9 TTCA	T 40 CACC GTGG T A AI 20 ACTC	H CCTC GAG L	T C FCA 850 CATGA STACT M TYPE_ 930 ACGTG	P C1 O ICT AGA I O GAC		P C A AI E CGGA R CGTC CGTC	P LLOI GGG T 940 GGGG	A YPE CTG GAC P	P AGG ICC E	87( TC/ AG! V 95( 2AA)	E AACA IGT T 0 0			G> 244 880 RGGT ACCA V V> 27 960 ACAA
810 GGACCGTCAGTCT CCTGGCAGTCAGAJ G P S V 1 890 GGTGGACGTGAGCG CCACCTGCACTGCACTCG	ICCIN AGGAO F L CACG	820 CTTC GAAC F 900 AAGJ TTC	P ACCC	8 CCAA GGTT P 9 TGAG	30 AACC TIGG K F FC 10 GTCA	> D  CCAA SGT1    	K 8 AGGA ICCT K D 1 - 9 TTCA AAGT	T 40 CACC GTGC T A AI 20 ACTC TGAC	H CCTC GAG L LLOI GGTZ	T C FCA 850 CATGA STACTJ M STACTJ M STACTJ M STACTJ 930 ACGTG NGCACO	P C1 0 ICT AGA I 0 GAC CTG		A AI E CGGZ R CGTC	P LLOI 360 CCCC T GGG T 940 GGAG CCTC	A YPE GAC P	P AGG ICC E CAT	1 87( TC) AG: V 95( 2AA:	E ACA IGT T 0 IGC ACG	L TGC ACC C	L CG: GCJ AGJ	G> 244 
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4G I'C	GCI CGA G	TCT. AGA F	0 ATC TAG Y	CCJ GGI P		FACA	TCG AGC I	CCG/ GGC/ A \	IGG ACC V	AGT TC/ E _FC/		GAGA CTCI E - i >:	AGCZ ICG S A A I>C	AATC TTAC N LLO	GGC CCG G TYPE	AGC TCG Q	CGC GCC P	BAGI E	ACI PTG: N	AACT PTG# N	TACJ ATG' Y	AAG ITC K	TGC TGC	АСС ЛСС Т	CCTX CGGA P	2CC 3GG P> 404
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	GCT CGA G CTC CGAC L	121 TCT. AGA F 129 GGAC CCTG D	0 ATC TAG Y 0 TCC S	CGA( GGT)	12 12 12 12 12 12 12 12 12 12 12 12 12 1	ACA EACA D 300 CTCC EAGC S	TCG AGC I TTC SAAC F	CCG GGCi A 1 TTC EAAG F	IGG ACC V 310 CTC GAC L	E FCA FCA TATA SATA Y FCA	rgg ACC W ΔC1 IAC ATC S	GAGA CTCT E 13: CCAA CGTT S K 1	AGC2 ICG S A A I>C I 20 GGA L A A	AATC TTAC N LLO CACC GTG T LLO	GGGC G IYPPE 13 CGTG GCAC V TYPE	AGC TCG Q : GAC CTC D :	CGC GCC P  XAA4 XTTV K	BAGI E E GAG CTC S	N 134 CAG GTC R	AACI M N GIGG GIGG CACC W	ACJ ATG Y SCA	AAG ITC K 13 GCA CGI	ACC TGC T 50 GGC 2 ( ( ( ( ( ( ( ( ( ( ( ( ( ( ( ( ( (	ACG TGC T GGAA CCT: 3 1 	CCTM CGGAM P 1 1 ACGT NGCA V V VotI	CCC 3GG P> 404 360 CTT GAA F> 43 
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PEIIHMTEGRELVIPCRVTS> $41 _ 45 _ bFIJTIIGDOMAIN 2 _ 55 _ 60001902002102202302400********CCTACT GTTACT TTA AAA AAGTIT CCA CTTGAC ACT TTG ATC CCTGAT GGA AAASCA TTG TAG TGA CAA TGA AATTTT TTCAAA GGT GAA ACTGGA AAC TAG GGA CTA CCTTTTPNITVTLKKFPDTIPDGK>6165bFIJTIIGDOMAIN 27580800300******************************************************************************************************$	GGG	CIT	TAA	TAT	GIG	TAC	TGA	CTT	CCT	TCC	CIC	GAG	CAG	TAA	GGG	ACG	GCC	CAA	TGC	AGT
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CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA GAA ATAGCG TAT TAG ACC CTG TCA TCT TTC COG AAG TAG TAT AGT TTA CGT TGC ATG TTT CTT TATRIIWDSRKGFIISNATYKEI>81			2	*			*			*			-	*			*			*
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-											D 232	ĸ	т	H _hFC	т AC1 л	C A	P	P	C> _240>
		7	30			740			750 *			7	60 *			770 *			780 *
CCA GGT P 241	GCA CGT A	CCT GGA P	GAA CIT E	CIC GAG L 245	CIG GAC L	666 CCC G	GGA CCT G	CCG GGC P hi	TCA AGT S FCAC	GTC CAG V	TTC AAG F	CTC GAG L	TIC AAG F	COC GGG P 255	GGT P	AAA TTT K	CCC GGG P	AAG TTC K	GAC CIG D> _260>
_		7	90			800			810			8	20			830			840
ACC TGG T 261	CTC GAG L	ATG TAC M	ATC TAG I	TCC AGG S _265	CGG GCC R	ACC TGG T	CCT GGA P	GAG CIC E h	GTC CAG V FCAC	ACA TGT T	TGC ACG C	GTG CAC V	GTG CAC V	GTG CAC V _275	GAC CTC D	GIG CAC V	AGC TCG S	CAC GTG H	GAA CTT E> _280>
		8	50			860 *			870 *			8	80 *			890 *			900 *
GAC CTG	CCT GGA	GAG	GTC	AAG	TTC	AAC	TGG ACC	TAC	GIG CAC	GAC		GIG	GAC	G GTO CAO	G CA1	CAA 1 XITI A		AAG TIC	ACA TGT
281		Е	v	к _285	F.	N	w	¥ h	FCAC	1 A	G	v	E	_295	п 	IN	ж 		_300>

Fig.24C.

		91	10		9	20			930			94	10		9	50			960
AAG	CCG	CGG	GAG	GAG	CAG	TAC	AAC	AGC	ACG	TAC	CGT	GIG	GTC	ÁGC	GTC	CIC	ACC	GIC	CTG
TTC	GGC	GCC	CIC	CIC	GIC	ATG	TTG	TCG	TGC	ATG	GCA	CAC	CAG	TCG	CAG	GAG	TGG	CAG	GAC
ĸ	Ρ	R	Е	Е	Q	Y	Ν	S	т	Y	R	v	v	S	v	L	т	v	L>
301_				_305_				h	CAC1	Α_				315_					_320>
		97	0 *		9	80 *			990 *			100	0 *		10	10		1	.020
CAC	CAG	GAC	TGG	CTG	ААТ	GGC	AAG	GAG	TAC	AAG	TGC	AAG	GIC	TCC	AAC	ААА	GCC	CIC	CCA
GTG	GIC	CIG	ACC	GAC	TTA	CCG	TIC	CIC	ATG	TIC	ACG	TIC	CAG	AGG	TTG	ттт	CGG	GAG	GGT
н	Q	D	W	$\mathbf{L}$	N	G	К	E	Y	К	С	к	v	S	N	ĸ	Α	L	<b>P&gt;</b>
321_				_325_				}	TCAC	1 A	<del></del>			_335_					_340>
		103	30 *		10	)40 *		-	L050 *			108	50 *		10	)70 *			1080 *
GCC	œ	ATC	GAG	AAA	ACC	ATC	TCC	ааа	GCC	AAA	GGG	CAG	ccc	CGA	GAA	CCA	CAG	GTG	TAC
œG	œG	TAG	CIC	TTT	TGG	TAG	AGG	TTT	CCGG	TTT	ccc	GIC	GGG	GCT	$\mathbf{CTT}$	GGT	GIC	CAC	ATG
Α	Р	I	Е	к	т	I	S	ĸ	Α	к	G	Q	P	R	Е	P	Q	v	Y> -
341_				_345_				h	FCAC1	A _				_355_					_360>
		109	90 *		1:	100 *		:	1110			112	20		11	L30 *		:	1140 *
ACC	CIG	ccc	CCA	TCC	œG	GAT	GAG	CIG	ACC	AAG	AAC	CAG	GIC	AGC	CIG	ACC	TGC	CIG	GIC
TGG	GAC	GGG	GGT	AGG	GCC	CTA	CIC	GAC	TGG	TIC	TIG	GIC	CAG	TCG	GAC	IGG	ACG	GAC	CAG
T	L	Ρ	P	S	R	D	Ε	L	т	K	N	Q	v	S	L	т	С	L	V>
361				_365_				h	FCAC1	Α_				_375_					_380>
		11!	50 *		1:	160 *		:	1170 *			11	B0		13	190 *			1200
ААА	GGC	TTC	TAT	ccc	AGC	GAC	ATC	GCC	GIG	GAG	TGG	GAG	AGC	AAT	GGG	CAG	CCG	GAG	AAC
TIT	CCG	AAG	ATA	GGG	TCG	CTG	TAG	CGG	CAC	CIC	ACC	CIC	TCG	TTA	œc	GIC	GGC	CIC	TIG
ĸ	G	F	Y	Ρ	S	D	I	A	v	Е	W	Е	S	N	G	Q	P	Е	N>
381				_385_				h	FCAC1	Α_	·			_395_					_400>
		12	10 *		1	220			1230 *			12	40 *		1	250 *			1260 *
AAC	TAC	AAG	ACC	ACG	CCT	ccc	GIG	CIG	GAC	тсс	GAC	GGC	TCC	TIC	TIC	CIC	TAC	AGC	AAG
TTG	ATG	TIC	TGG	TGC	GGA	GGG	CAC	GAC	CIG	AGG	CIG	CCG	AGG	AAG	AAG	GAG	ATG	TCG	TIC
N	Y	к	т	T	Р	Р	v	Ŀ	D	S	D	G	S	F	F	L	Y	S	K>
401				_405				n	FCACI	. А.				_415_					_420>
		12	70 *		1	280 *			1290 *			13	00 *		1	310 *			1320 *
стс	ACC	GIG	GAC	AAG	AGC	AGG	TGG	CAG	CAG	GGG	AAC	GTC	TIC	TCA	TGC	TCC	GIG	ATG	CAT
GAG	TGG	CAC	CIG	TIC	TCG	TCC	ACC	GIC	GIC	ccc	TIG	CAG	AAG	AGT	ACG	AGG	CAC	TAC	GTA
L	т	v	D	K	s	R	W	Q	Q	G	N	v	F	S	С	S	v	М	H>
421				_425				h	FCAC1	LA.				_435_					_440>
		13	30 *		1	340 *			1350 *			13	60 *		1	370 *			
GAG	GCT	CIG	CAC	AAC	CAC	TAC	ACG	CAG	AAG	AGC	CTC	TCC	CIG	TCT	CCG	GGT	ААА	TGA	1
CIC	CGA	GAC	GIG	TTG	GIG	ATG	TGC	GIC	TTC	TOG	GAG	AGG	GAC	AGA	GGC	CCA	TTT	ACT	2
E	A	L	н	N	н	Y	т	Q	K	S	L	S	$\mathbf{L}$	S	Р	G	K	*>	•
441				_445				_hFC	∆C1 /	۹				_455			_458		>

Fig.25A.









May 20, 2008





/2Flk1D3.FcΔC1(a) & VEGFR1R2-FcΔC1(a)	VEGF/VEGFR1R2-FcΔC1(a)	0.98	0.94	0.99	0.97 ± 0.02	
oichiometry of hVEGF165 to Flt1E	VEGF/FIt1D2FIk1D3.FcΔC1(a)	0.93	0.97		0.96 ± 0.03	
Binding St	hVEGF165 (nM)	-	10	50	Average ± StDev	

Fig.28.

Fig.29. 1E-09 Free Flt1D2Flk1D3.FcΔC1(a) (M) 1E-09 Y Intercept = 0.989 nM X Intercept = 0.936 nM 8E-10 Slope = -1.065E-10 3E-10 0E+00 (2E-10) 5E-10 0E+00 1E-09 2E-09 2E-09 3E-09 3E-09 Total VEGF Added (M) Fig.30. 1E-09 Free VEGFR1R2-FcdC1(a) (M) Y Intercept = 0.988 nM 1È-09 X Intercept = 0.926 nM Slope = -1.07 8E-10 5E-10 3E-10 0E+00 (2E-10) 1E-09 5E-10 0E+00 2E-09 2E-09 3E-09 3E-09 Total VEGF Added (M)







Fig.36.
GRPFVEMYSEIPEIIHMTEGRELVIPCRVTSPNITVTLKKFPLDTLIPDG
KRIIWDSRKGFIIS <u>N</u> ATYKEIGLLT <u>C</u> EATVNGHLYKTNYLTHRQTNTIID
VVLSPSHGIELSVGEKLVL <u>NC</u> TARTELNVGIDFNWEYPSSKHQHKKLVNR
DLKTQSGSEMKKFLSTLTIDGVTRSDQGLYTCAASSGLMTKKNSTFVRVH
EKGPGDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD
VSHEDPEVKFNWYVDGVEVHNAKTKPREEQY <u>N</u> STYRVVSVLTVLHQDWLN
GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL
TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS
RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK









## MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES

This application is a divisional of U.S. patent application 5 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 10 applications are herein specifically incorporated by reference in their entirety.

## 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 a way as to improve their pharmacokinetic profile. The field of this invention also relates to 20 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, survival, cell product secretion, or differentiation is often 34 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 the protein with its ligand binding characteristic. 35 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 of this catalytic intracellular domain 40 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 kinases (RTKs) such as the Trk family 45 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 50 coupled receptors such as the  $\beta_2$ -adrenergic receptor found on, for instance, cardiac muscle cells, and the multimeric IgE high affinity receptor  $Fc \in RI$  which is localized, for the most part, on mast cells and basophils (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, Neuoron 9:383-391) and molecular interactions between dimerizing intracellular domains lead to activation of catalytic function. In some instances, such as platelet-derived growth factor (PDGF), the ligand is a dimer that binds two receptor molecules (Hart, et al., 1988, Science, 240:1529-1531; Hel-65 din, 1989, J. Biol. Chem. 264:8905-8912) while, for example, in the case of epidermal growth factor (EGF), the

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

Often, the tissue distribution of a particular receptor within higher organisms provides insight into the biological function of the receptor.

The RTKs for some growth and differentiation factors, such as fibroblast growth factor (FGF), are widely expressed and therefore appear to play some general role in tissue growth and maintenance. Members of the Trk RTK family (Glass & Yancopoulos, 1993, Trends in Cell Biol. 3:262-268) of receptors are more generally limited to cells of the nervous system, and the Nerve Growth Factor family consisting of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), which bind the Trk RTK family receptors, promote the differentiation of diverse groups of neurons in the brain and periphery (Lindsay, R. M, 1993, in Neurotrophic Factors, S. E. Loughlin & J. H. Fallon, eds., pp. 257-284, San Diego, Calif., Academic Press). FceRI is localized to a very limited number of types of cells such as 25 mast cells and basophils. Mast cells derive from bone marrow pluripotent hematopoietic stem cell lineage, but complete their maturation in the tissue following migration from the blood stream (See Janeway & Travers, 1996, in Immunobiology, 2d. Edition, M. Robertson & E. Lawrence, eds., pp. 1:3-1:4, Current Biology Ltd., London, UK, Publisher) and are involved in the allergic response.

Many studies have demonstrated that the extracellular domain of a receptor provides the specific ligand binding characteristic.

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

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

The membrane-bound tyrosine kinase receptor, known as Flt, was shown to be a VEGF receptor [DeVries, C. et al., (1992), Science, 255, pp.989-991]. The Flt receptor specifically binds VEGF which induces mitogenesis. Another form of the VEGF receptor, designated KDR, is also known to bind VEGF and induce mitogenesis. The partial cDNA sequence and nearly full length protein sequence of KDR is known as well [Terman, B. I. et al., (1991) Oncogene 6, pp. 1677-1683; Terman, B. I. et al., (1992) Biochem. Biophys. Res. Comm. 187, pp. 1579-1586].

Persistent angiogenesis may cause or exacerbate certain 5 diseases such as psoriasis, rheumatoid arthritis, hemangiomas, angiofibromas, diabetic retinopathy and neovascular glaucoma. An inhibitor of VEGF activity would be useful as a treatment for such diseases and other VEGF-induced pathological angiogenesis and vascular permeability conditions, such as tumor vascularization. The present invention relates to a VEGF inhibitor that is based on the VEGF receptor Flt1.

Plasma leakage, a key component of inflammation, occurs in a distinct subset of microvessels. In particular, in most organs plasma leakage occurs specifically in the venules. Unlike arterioles and capillaries, venules become leaky in response to numerous inflammatory mediators including histamine, bradykinin, and serotonin. One characteristic of inflammation is the plasma leakage that results from inter- 20 cellular gaps that form in the endothelium of venules. Most experimental models of inflammation indicate that these intercellular gaps occur between the endothelial cells of postcapillary and collecting venules (Baluk, P., et al., Am. J. Pathol. 1998 152:1463-76). It has been shown that certain 25 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 lectins have been used to visualize morphological changes at 30 endothelial cell borders in inflamed venules of, for example, the rat trachea. Lectins, such as conconavalin A and ricin, that bind focally to inflamed venules reveal regions of the subendothelial vessel wall exposed by gaps that correspond to sites of plasma leakage (Thurston, G., et al., Am J Physiol, 35 1996, 271: H2547-62).

The properties of the microvessels are dynamic. Chronic inflammatory diseases, for example, are associated with microvascular remodeling, including angiogenesis and microvessel enlargement. Microvessels can also remodel by 40 acquiring abnormal phenotypic properties. In a murine model of chronic airway inflammation, airway capillaries acquire properties of venules, including widened vessel diameter, increased immunoreactivity for von Willebrand factor, and increased immunoreactivity for P-selectin. In 45 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 vascular permeability and/or plasma leakage. For example, 50 mystixins are synthetic polypeptides that have been reported to inhibit plasma leakage without blocking endothelial gap formation (Baluk, P., et al., J. Pharmacol. Exp. Ther., 1998, 284: 693-9). Also, the beta 2-adrenergic receptor agonist formoterol reduces microvascular leakage by inhibiting 55 endothelial gap formation (Baluk, P. and McDonald, D. M., Am. J.

Physiol., 1994, 266:L461-8).

The angiopoietins and members of the vascular endothelial growth factor (VEGF) family are the only growth factors 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 remodeling. ⁶⁵

U.S. Pat. No. 6,011,003, issued Jan. 4, 2000, in the name of Metris Therapeutics Limited, discloses an altered, soluble

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form of FLT polypeptide being capable of binding to VEGF and thereby exerting an inhibitory effect thereon, the polypeptide comprising five or fewer complete immunoglobulin domains.

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

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

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

PCT Publication No. WO 97/13787, published Apr. 17, 1997, in the name of 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 immunoglobulin-like domain and the second immunoglobulin-like domain in the extracellular region of a VEGF receptor FLT but not containing the sixth immunoglobulin-like domain and the seventh immunoglobulin-like domain thereof shows a VEGF inhibitory activity.

Sharifi, J. et al., 1998, The Quarterly Jour. of Nucl. Med. 42:242-249, disclose that because monoclonal antibodies (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 itself.

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

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

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

R. Duncan and F. Spreafico, Clin. Pharmacokinet. 27: 290-306, 296 (1994) review efforts to improve the plasma 65 half-life of asparaginase by conjugating polyethylene glycol.

PCT International Publication No. WO 99/03996 published Jan. 28, 1999 in the name of Regeneron Pharmaceuticals, Inc. and The Regents of The University of California describes modified human noggin polypeptides having deletions of regions of basic amino acids. The modified human noggin polypeptides are described as retaining biological activity while having reduced affinity for heparin and superior pharmacokinetics in animal sera as compared to the unmodified human noggin.

## SUMMARY OF THE INVENTION

The present invention is directed to VEGF antagonists with improved pharmacokinetic properties. A preferred embodiment is an isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a VEGF polypeptide comprising (a) a nucleotide sequence encoding a VEGF receptor component operatively linked to (b) a nucleotide sequence encoding a multimerizing component, wherein the VEGF receptor component is the only VEGF receptor component of the fusion polypeptide and wherein the nucleotide sequence of (a) consists essentially of a nucleotide 20 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 second VEGF receptor is Flk1.

In yet another embodiment, the isolated nucleic acid of  $_{30}$  the second VEGF receptor is Flt4.

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

In still another preferred embodiment, the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain 40 of the second VEGF receptor.

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

In another embodiment, the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, 45 the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include an isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding 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-14**C (SEQ ID NO:5);
- (c) the nucleotide sequence set forth in FIG. **15A-15**C (SEQ ID NO:7);
- (d) the nucleotide sequence set forth in FIG. **16A-16**D (SEQ ID NO:9);
- (e) the nucleotide sequence set forth in FIG. **21A-21**C ₆₀ (SEQ ID NO:11);
- (f) the nucleotide sequence set forth in FIG. **22**A-**22**C (SEQ ID NO:13),
- (g) the nucleotide sequence set forth in FIG. **24**A-**24**C (SEQ ID NO:15); and
- (h) a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucle-

otide sequence of (a), (b), (c), (d), (e), (f), or (g) and which encodes a fusion polypeptide molecule having the biological activity of the modified Flt1 receptor 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 ¹⁰ comprising a multimer of the fusion polypeptide.

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

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

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

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

Additional embodiments include a fusion polypeptide encoded by the nucleic acid sequence set forth in FIG. **10A-10**D (SEQ ID NO:1) or FIG. **24A-24**G (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 specifically binds the VEGF receptor ligand VEGF.

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

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

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

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

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

In a further embodiment of the fusion polypeptide, the 10 first VEGF receptor is Flt1.

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

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

Preferred embodiments include a fusion polypeptide 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 polypep- 20 tide wherein the amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor.

In yet another embodiment, the fusion polypeptide mul- 25 timerizing component comprises an immunoglobulin domain including an embodiment wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include a fusion polypeptide 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 35 forth in FIG. 14A-14D (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-21D (SEQ ID NO:12); (f) the amino acid sequence set 40 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 45 described above.

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

## BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. IEF gel analysis of unmodified and acetylated Flt1(1-3)-Fc proteins. Unmodified Flt1(1-3)-Fc protein is 55 unable to enter the gel due to its >9.3 pl, whereas acetylated Flt1(1-3)-Fc is able to enter the gel and equilibrate at pl 5.2.

FIG. 2. Binding of unmodified Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc proteins to MATRIGEL® coated plates. Unmodified Flt1(1-3)-Fc proteins binds extensive to extra- 60 cellular matrix components in Matrigel.RTM., whereas acetvlated Flt1(1-3)-Fc does not bind.

FIG. 3. Binding of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc in a BIACORE[™]based assay. Acetylated (columns 13-16), pegylated (col- 65 umns 17-20), and heparin-treated Flt1(1-3)-Fc (columns 21-24) are each able to completely compete 25 with the

BIACORE™ chip-bound Flt1(1-3)-Fc for VEGF binding as compared to control (columns 1-4) and irrelevant protein (columns 5-8). Unmodified Flt1(1-3)-Fc (columns 5-6) appears to only partially compete with BIACORETM chipbound Flt1(1-3)-Fc for VEGF binding. However, washing the bound samples with 0.5M 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 Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc to VEGF in an ELISA-based assay. Both pegylated and acetylated Flt1(1-3)-Fc proteins bind to VEGF with affinities approaching that of unmodified Flt1(1-3)-Fc.

FIG. 5. Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc. Balb/c mice (23-28 g) were injected subcutaneously with 4 mg/kg of unmodified, acetylated, or pegylated Flt1(1-3)-Fc. The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days, and 3 days after injection of protein and the sera were assayed in a standard ELISA-based assay designed to detect Flt1(1-3)-Fc protein. The  $T_{max}$  for all of the Flt1(1-3)-Fc proteins was between the 6 hour and 24 hour time points. The  $C_{max}$  for the different proteins was as follows: Unmodified: 0.06 µg/ml-0.15 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 stepacetylated Flt1(1-3)-Fc proteins. Unmodified Flt1(1-3)-Fc protein is unable to enter the gel due to its >9.3 pl, 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 pls ranging between 4.55-8.43, depending on the degree of acetylation.

FIG. 7. Binding of unmodified Flt1(1-3)-Fc and stepacetylated Flt1(1-3)-Fc proteins to MATRIGEL® coated plates. As with the irrelevant control protein, rTie2-Fc, 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 binding to extracellular matrix components.

FIG. 8. Binding of unmodified Flt1(1-3)-Fc and stepacetylated Flt1 (1-3)-Fc in a BIACORETM-based 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 50 or step-acetylated) 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(1-3)-Fc protein (either unmodified or step-acetylated) to completely saturate the available VEGF. However, at 5.0 µg/ml, which is several times greater than a 1:1 stoichiometric ratio, both the Flt1(1-3)-Fc and the step-acetylated Flt1(1-3)-Fc proteins are able to saturate the VEGF, regardless of the degree of acetylation.

FIG. 9. Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc. Balb/c mice (23-28 g) were injected subcutaneously with 4 mg/kg of unmodified or 10, 20, 40, 60 and 100 fold excess samples of stepacetylated 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  $\mu$ g/ml; 10 fold excess sample:—0.7  $\mu$ g/ml, 20 fold excess sample—2  $\mu$ g/ml, 40 fold excess sample—4  $\mu$ g/ml, 60 fold excess sample—2  $\mu$ g/ml, 100 fold excess sample—1  $\mu$ g/ml.

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

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

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

FIG. **13A-13**D. 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-14D**. Nucleic acid (SEQ ID NO:5) and deduced amino acid sequence (SEQ ID NO:6) of Mut2-Flt1( $2-3_{\Delta^{B}}$ )-Fc.

FIG. **15A-15**C. Nucleic acid (SEQ ID NO:7) and deduced amino acid sequence (SEQ ID NO:8) of Mut3: Flt1 (2-3)-Fc. 20

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

FIG. 17. Binding of unmodified Flt1(1-3)-Fc, basic region deletion mutant Flt1(1-3)-Fc, and Flt1(1-3)_{$R \rightarrow N$} mutant pro- 25 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 BIACORE[™] chip. 30 At 0.5 µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, the stoichiometric ratio approximates 1:1 and there is an increased ability to block VEGF binding to the BIACORE™ chip. At 1.0 µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc pro- 35 teins, which is approximately a 10:1 stoichiometric ratio, the Flt1(1-3)-Fc proteins are able to block binding of VEGF to the BIACORETM 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: 40 Flt1(1-3_{*R* $\rightarrow$ *N*)-Fc is somewhat less efficient at blocking bind-} ing.

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 45 protein binds avidly to these wells, the Mut3: Flt1(2-3)-Fc protein binds somewhat more weakly, the Mut1: Flt1(1- $3_{\Delta B}$ )-Fc protein binds more weakly still, and the Mut2: Flt1( $2-3_{\Delta B}$ )-Fc protein shows the best profile, binding more weakly than any of the other mutant proteins. The Mut4: 50 Flt1( $1-3_{R \to N}$ )-Fc glycosylation mutant protein shows only marginal benefit 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 $(2-3_{\Delta B})$ -Fc, and Flt1(2-3) mutant proteins bind VEGF similarly.

FIG. **20**. Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1( $1-3_{\Delta B}$ )-FC, Mut2: F6ti ( $2-3_{\Delta B}$ )-Fc, and 60 Flt1(2-3) mutant proteins. the Cmax for these reagents was as follows: Unmodified Flt1(1-3)-Fc-0.15 µg/ml; 40 fold molar excess acetylated Flt1(1-3)-Fc-1.5 µg/ml; and Mut1: Flt1( $1-3_{\Delta B}$ )-Fc-0.7 µg/ml.

FIG. **21A-21D**. Nucleotide (SEQ ID NO:11) and deduced 65 amino acid sequence (SEQ ID NO:12) of the modified Flt1 receptor termed Flt1D2.Flk1D3.Fc $\Delta$ C1(a).

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

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

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

FIG. **25**A-**25**C. Phosphorylation assay. At a 1.5 molar excess of either Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3.Fc $\Delta$ C1(a) there is complete blockage of receptor stimulation by these three modified Flt1 receptors as compared to control media challenge. In contrast, transient Flt1D2VEGFR3D3.Fc $\Delta$ C1(a) does not show significant blockage at this molar excess, as compared with VEGF positive control challenge. Similar results are seen in FIG. **25**B, where the modified Flt receptors are in a 3-fold molar excess to VEGF165 ligand. In FIG. **25**C, where the modified Flt1 receptors are in a 6-fold molar excess to VEGF165 ligand, transient Flt1D2VEGFR3D3.Fc $\Delta$ C1(a) can now be shown to be partially blocking VEGF165-induced stimulation of cell-surface receptors.

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

FIG. 27. MG/R2 Cell proliferation assay. The following modified Flt receptors Flt1(1-3)-Fc, Flt1D2.Flk1D3.Fc $\Delta$ C1 (a) and Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a), plus an irrelevant receptor termed Tie2-Fc as a negative control, were titrated from 40 nM to 20 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 VEGF165-induced cell proliferation at any concentration whereas Flt1D2.Flk1D3.Fc $\Delta$ C1(a) blocks 1.56 nM VEGF165 with a half maximal dose of 0.8 nM. Flt1(1-3)-Fc and Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a) are less effective in blocking VEGF165 in this assay with a half maximal dose of -2 nM. VEGF165 alone gives a reading of 1.2 absorbance units and the background is 0.38 absorbance units.

FIG. **28**. BIACORETM analysis of Binding Stoichiometry. Binding 20 stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1D2Flk1D3.Fc. $\Delta$ C1 (a) or VEGFR1R2-Fc $\Delta$ C1(a), using the conversion factor of 1000 RU equivalent to 1 ng/ml. The results indicated binding stoichiometry of one VEGF165 dimeric molecule per one Flt1D2Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a) molecule.

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

FIG. 31. Size Exclusion Chromatography (SEC) under native conditions. Peak #1 represents the Flt1D2Flk1D3.Fc\DeltaC1(a)/VEGF165 complex and peak #2 10 represents unbound VEGF165. Fractions eluted between 1.1 and 1.2 ml were combined and guanidinium hydrochloride (GuHCl)was added to a final concentration 4.5M to dissociate the complex.

dissociative conditions. To separate the components of the receptor-ligand complex and to determine their molar ratio, 50.mu.l of dissociated complex was loaded onto a SUPER-OSE™ 12 PC 3.2/30 equilibrated in 6M GuHCl and eluted. Peak#1 represents Flt1D2Flk1D3.FcΔC1(a) and peak#2 rep- 20 resents VEGF165.

FIG. 33, FIG. 34 and FIG. 35. Size Exclusion Chromatography (SEC) With On-Line Light Scattering. Size exclusion chromatography column with a MiniDawn on-line light scattering detector (Wyatt Technology, Santa Barbara, 25 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 30 unbound VEGF165. MW was calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows:

MW of the Flt1D2Flk1D3.Fc∆C1(a)NEGF165 complex 35 at the peak position is 157 300 (FIG. 33), the MW of VEGF165 at the peak position is 44 390 (FIG. 34) and the MW of R1R2 at the peak is 113 300 (FIG. 35).

FIG. 36. Peptide mapping and glycosylation analysis. The disulfide structures and glycosylation sites in 40 Flt1D2.Flk1D3.Fc\DeltaC1(a) (SEQ ID NO:12) were determined by a peptide mapping method. There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 is disulfide bonded to Cys76. Cys121 is disulfide bonded to Cys 182. The first two cysteines in the 45 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 Cys211. 50 Cys216 is disulfide bonded to Cys306. Cys 352 is disulfide bonded to Cys410.

There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.Fc $\Delta$ C1(a) (SEQ ID NO:12) and are found to be glycosylated to varying degrees. Complete glycosylation 55 is observed at Asn33, Asn193, and Asn282. Partial glycosylation is observed on Asn65 and Asn120. Sites of glycosylation are highlighted by underline in the FIG.

FIG. 37. Pharmacokinetics of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and VEGFR1 R2-Fc $\Delta$ C1(a). 60 Balb/c mice were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed stably Flt1D2.Flk1D3.Fc $\Delta$ C1(a), CHO expressed Flt1D2.Flk1D3.Fc $\Delta$ C1(a), and CHO transiently expressed VEGFR1R2-Fc $\Delta$ C1(a). The mice were tail bled at 1, 2, 4, 6, 65 24 hrs, 2 days, 3 days and 6 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc

(A40), Flt1D2.Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a). The Tmax for Flt1(1-3)-Fc (A40) was at 6 hrs while the Tmax for the transient and stable Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and the transient VEGFR1R2-Fc\DeltaC1(a) was 24 hrs. The Cmax for Flt1(1-3)-Fc (A40) was 8 µg/ml, For both transients (Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and VEGFR1R2-Fc $\Delta$ C1 (a)) the Cmax was 18 µg/ml and the Cmax for the stable VEGFR1R2-FcΔC1(a) was 30 µg/ml.

FIG. 38. Pharmacokinetics of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1 (a). Balb/c mice were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO 5 transiently expressed Flt1D2.Flk1D3.Fc\DeltaC1(a) and CHO transiently expressed Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a). The mice were tail bled at 1, FIG. 32. Size Exclusion Chromatography (SEC) under 15 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc, Flt1D2.Flk1D3.Fc\DeltaC1(a) and Flt1D2.VEGFR3D3.Fc\DeltaC1 (a). Flt1(1-3)-Fc (A40) could no longer be detected in the serum after day 5 whereas Flt1D2.Flk1D3.FcΔC1(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. 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.

FIG. 42A-42B. Assessment of Corpus Luteum Angiogenesis Using Progesterone as a Readout. PMSG was injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats, resulting in a fully functioning corpus luteum containing a dense network of blood vessels that secretes progesterone into the blood stream to prepare the uterus for implantation. The induction of angiogenesis in the corpus luteum requires VEGF. Resting levels of progesterone are about 5 ng/ml and can be induced to 25-40 ng/ml after PMSG. Subcutaneous injection of Flt1(1-3)-Fc (A40) or Flt1D2.Flk1D3.Fc\DeltaC1(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 5 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 10 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, 20 valine, isoleucine, 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 25 lysine or arginine. von Heijne has published detailed rules that are commonly referred to by skilled artisans when determining which amino acids of a given receptor belong to the extracellular, transmembrane, or intracellular domains (See von Heijne, 1995, BioEssays 17:25-30). Alternatively, 30 websites on the Internet have become available to provide protein chemists with information about making predictions about protein domains.

The present invention provides for the construction of nucleic acid molecules encoding chimeric polypeptide mol- 35 ecules that are inserted into a vector that is able to express the chimeric polypeptide molecules when introduced into an appropriate host cell. Appropriate host cells include, but are not limited to, bacterial cells, yeast cells, insect cells, and mammalian cells. Any of the methods known to one skilled 40 in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding the chimeric polypeptide molecules under control of transcriptional/translational control signals. These methods may include in vitro recombinant DNA and synthetic techniques 45 and in vivo recombinations (genetic recombination) (See Sambrook, et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory; Current Protocols in Molecular Biology, Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY).

Expression of nucleic acid molecules encoding the chimeric polypeptide molecules may be regulated by a second nucleic acid sequence so that the chimeric polypeptide molecule is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the 5 chimeric polypeptide molecules described herein may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control expression of the chimeric polypeptide molecules include, but are not limited to, the long terminal repeat as described in Squinto 60 et al., (1991, Cell 65:1-20); the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the CMV promoter, the M-MuLV 5' terminal repeat the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory

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sequences of the metallothionine gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the  $\beta$ -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, 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, 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 Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Shani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

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

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

containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional propserties 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 10 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 quan-15 titatively by 8M guanidinium hydrochloride and dialysis (see, for example, Builder, et al., U.S. Pat. No. 5,663,304). In order to further purify the factors, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration 20 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 25 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' 35 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 40 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 45 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 50 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 55 and pleural effusion associated with tumors, inflammation or trauma; chronic airway inflammation; capillary leak syndrome; sepsis; kidney disease associated with increased leakage of protein; and eye disorders such as age related macular degeneration and diabetic retinopathy. 60

An amino acid sequence analysis of Flt1(1-3)-Fc revealed the presence of an unusually high number (46) of the basic amino acid residue lysine.

An IEF analysis of Flt1(1-3)-Fc showed that this protein has pl greater than 9.3, confirming the prediction that the 65 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 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., 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 25 µM methionine sulfoximine (MSX) from Sigma Inc., St. Louis, Mo., and high recombinant protein expressors were obtained by screening the CHO K1 cell supernatants from over 100 hand-picked colony isolates using a standard immunoassay which captures and detects human Fc. The selected hand-picked clone was amplified in the presence of 100 µM 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 pg/cell/day.

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

#### Example 2

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

Flt1(1-3)-Fc protein was initially purified by affinity chromatography. A Protein A column was used to bind, with high specificity, the Fc portion of the molecule. This affinitypurified 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.times. concentrate from Life Technologies, Gaithersburg, Md. Protein A Fast Flow and SUPERDEXTM 200 preparation grade resins were obtained from Pharmacia, Piscataway, N.J. Equipment and membranes for protein concentration were obtained from Millipore, Bedford, Mass.

Approximately 40L of 0.45 µm-filtered CHO conditioned media containing Flt1(1-3)-Fc protein was applied to a 290 mL Protein A Fast Flow column (10 cm diameter) that had been equilibrated with PBS. The column was washed with PBS containing 350 mM NaCl and 0.02% CHAPS and the bound protein was eluted with 20 mM Citric Acid containing 10 mM Na₂HPO₄. The single peak in the elution was collected and its pH was raised to neutrality with 1M 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 25 remove aggregates and other contaminants, the concentrated protein was applied to a column packed with Superdex 200 preparation grade resin (10 cm×55 cm) and run in PBS containing 5% glycerol. The main peak fractions were pooled, sterile filtered, aliquoted and stored at -80° C.

#### Example 3

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

Two milligrams of Flt1 (1-3)-Fc protein were acetylated ³⁵ 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

(a)IEF analysis: 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 pl 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 pl of approximately 5.2. This result demonstrates that acetylation reduces the net positive charge of the protein and therefore its pl considerably.

## b. Binding to Extracellular Matrix Components

To test for binding to extracellular matrix components, 55 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 Matrigel (Biocoat MATRIGEL®) matrix thin layer 96 well plate, Catalog #40607, Becton 60 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. The plates are incubated for 1-2 hours at either room temperature or 37° C. degrees and 65 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 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 Flt1(1-3)-Fc, but rather by physically shielding the positive charges from interacting with the extracellular matrix. Applicants decided to attempt to improve the pharmacokinetic properties of Flt1(1-3)-Fc molecules by attaching strands of 20K PEGs as described infra.

#### Materials and Methods

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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 6 column from Pharmacia, Piscataway, N.J.; PBS as a 10x concentrate from Life Technologies, Gaithersburg, Md.; Glycerol from J. T. Baker, Phillipsburg, N.J.; and Bis-Tris precast gels from Novex, Calif.

20K PEG strands functionalized with amine-specific 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 reactions and the analyses of samples on standard SDS-PAGE, Flt1(1-3)-Fc at a concentration of 1.5 mg/mL was reacted at pH 8.1 with 20K SPA-PEG (PEG succinimidyl propionate) molecules at a PEG-to-Flt1(1-3)-Fc monomer molar ratio of 1:6. The reaction was allowed to proceed at 8° C. overnight. For initial purification, the reaction products were applied to a 10 mm×30 cm Superose 6 column equilibrated with PBS containing 5% Glycerol. The column appeared to separate pegylated Flt1(1-3)-Fc molecules based on the extent of pegylation. Fractions corresponding to what appeared to be primarily mono-pegylated and di-pegylated dimeric Flt1(1-3)-Fc, as judged by banding patterns on reducing and non-reducing SDS-PAGE gels were pooled. The protein concentration was determined by measuring absorbance at 280 nm. The pegylated Flt1(1-3)-Fc protein was sterile filtered, aliquoted and stored at  $-40^{\circ}$ C.

## Example 6

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

Unmodified, acetylated, and pegylated Flt1(1-3)-Fc proteins were tested in a BIACORETM-based assay to evaluate their ability to bind to the Flt1 ligand, VEGF. In this assay, unmodified Flt1(1-3)-Fc protein was immobilized on the surface of a BIACORETM chip (see BIACORETM Instruction 10 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 non-specific binding, the bound samples were washed with a 0.5M 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, 20 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 25 FIG. 3, 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 BIACORETM chip-bound Flt1(1-3)-Fc for VEGF binding as compared to control (columns 1-4) and irrelevant protein (columns 5-8). 30 Unmodified Flt1(1-3)-Fc (columns 5-6) appeared to only partially compete with BIACORE™ chip-bound Flt1(1-3)-Fc for VEGF binding. However, washing the bound samples with 0.5M NaCl (columns 7-8) resulted in a binding profile similar to the modified forms of Flt1(1-3)-Fc, indicating that 35 the unmodified protein was exhibiting non-specific binding to the chip that could be eliminated by the salt wash.

## Example 7

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

Unmodified, acetylated, and pegylated Flt1(1-3)-Fc proteins were tested in a standard ELISA-based assay to evalu-45 ate 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. 50

## 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(1-3)-Fc, and pegylated Flt1(1-3)-Fc protein. Balb/c 60 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 65 Flt1(1-3)-Fc protein. Briefly, the assay involves coating an ELISA plate with VEGF, binding the unmodified, acety-

lated, 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 Tmax for all of the Flt1(1-3)-Fc proteins was between the 6 hour and 24 hour time points. The Cmax for the different proteins was as follows: Unmodified:  $0.06 \ \mu$ /ml-0.15 µg/ml; acetylated:  $1.5 \ \mu$ g/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 kit (Pierce Chemical Co., Rockford, Ill, Cat.#26777).

#### Example 10

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

 (a 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. 6A-6B, unmodified Flt1(1-3)-Fc protein was not able to migrate into the gel due to its extremely high pl (greater than 9.3). However, most of the 35 step-acetylated Flt1(1-3)-Fc samples (30-100 fold molar excess 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

pl can be controlled by controlling the degree of acetylation.

(b.) 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-acety-50 lated 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 phosphatase-conjugated anti-human Fc antibody to the wells. Alkaline phosphatase substrate was subsequently added to the wells and optical density measured. FIG. 7 shows the results of this assay. Like the irrelevant control protein rTie2-Fc, step-acetylated Flt1(1-3)-Fc (20 and 30 fold molar excess samples) did not exhibit any significant binding to the Matrigel coated plate, whereas the nonacetylated 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.

(c.) Binding of Step-Acetylated Ftl1(1-3)-Fc in a BIA-CORETM-Based Assay  10 

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 BIACORE™ 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 1  $\mu$ g/ml VEGF), there is not enough Flt1(1-3)-Fc (either unmodified 25 or step-acetylated) 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 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 35 demonstrates that acetylation does not alter Flt1(1-3)-Fc's ability to bind VEGF.

(d.) Pharmacokinetic Analysis of Step-Acetylated Flt1(1-3)-Fc

In vivo experiments were designed to assess the pharma- 40 cokinetic profiles of unmodified Flt1(1-3)-Fc and stepacetylated 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 stepacetylated 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 50 study. The Tmax for all of the Flt1(1-3)-Fc proteins tested was at the 6 hour time point but the Cmax was as follows: Unmodified Flt1(1-3)-Fc: 0.06 µg/ml; 10 fold molar excess sample:-0.71 µg/ml, 20 fold molar excess sample-2 µg/ml, 40 fold molar excess sample—4 µg/ml, 60 fold molar 5. excess sample-2 µg/ml, 100 fold molar excess sample-1 µg/ml. This results demonstrates that acetylation or pegylation of Flt1(1-3)-Fc significantly improves its pharmacokinetic profile.

#### Example 11

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

Based on the observation that acetylated Flt1(1-3)-Fc, which has a pl below 6, has much better pharmacokinetics

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than the highly positive unmodified Flt1(1-3)-Fc (pl>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 surface 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 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 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 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 various 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 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 394 to 687 (encoding Gly-Arg-Pro to Asn-Thr-Ile), and Flt1 Ig domain 3 extends from nucleotides 688 to 996 (encoding Ile-Asp-Val to Asp-Lys-Ala). There is a bridging amino acid sequence, Gly-Pro-Gly, encoded by nucleotides 997-1005, followed by the nucleotide sequence encoding human Fc (nucleotides 1006-1701 or amino acids Glu-Pro-Lys to Pro-Glv-Lvs-stop).

A more detailed analysis of the Flt1 amino acid sequence reveals that there is a cluster, namely, amino acid residues 272-281 (KNKRASVRR) of 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 FlG. 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 (MaeVaster MACVECTORTM 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 60 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 receptor for VEGF. This DNA construct, which was constructed using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, F et

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al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, N.Y.) in the mammalian expression vector pMT21, (Genetics Institute, Inc., Cambridge, Mass.), is referred to as Mut1: Flt1(1-3 $_{\Delta B}$ )-Fc. The Mut1: 5 Flt1(1-3_{AB})-Fc constructwas 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 3. 10

The final DNA construct was sequence-verified using an ABI 373A DNA sequencer and Tag 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. 13A-13D.

#### 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, 25 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 Labora- 30 tory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, Mass.), was also sequence-veri- 35 fied 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- $3_{AB}$ )-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 50 techniques and the final construct was sequence-verified verified as described supra. The sequence of Mut3: Flt1(2-3)-Fc is set forth in FIG. 15A-15C.

#### Example 14

## Construction of Flt(1-3)-Fc Basic Region N-Glycosylation Mutant Designated Mut4: Flt1(1-3_{$R \rightarrow N$})-Fc

A final construct was made in which a N-glycosylation 60 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-10). The resulting amino acid sequence is therefore changed from Arg-Ala-Ser to Asn-Ala-Ser, which matches

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the canonical signal (Asn-Xxx-Ser/Thr) for the addition of a N-glycosylation site at the Asn residue. The sequence of Mut4: Flt1(1-3_{$R \rightarrow N$})-Fc is set forth in FIG. 16A-16D.

#### Example 15

## Characterization of Acetylated Flt1(1-3)-Fc, Mut1: Flt1(1-3_{$\Delta B$})-Fc, and Mut4: Flt1(1-3_{$R \rightarrow N$})-Fc Mutants

(a.) Binding to Extracellular Matrix Components

To determine whether the three modified proteins were more or less likely to have improved pharmacokinetic 15 properties, Matrigel coated 96-well dishes (as described supra) were incubated with varying concentrations of the mutant proteins and detected with anti-human Fc/alkalinephosphatase conjugated antibodies. As shown in FIG. 18, this experiment showed that while the unmodified Flt1(1-3)-Fc protein could bind avidly to these wells, the Mut3: Flt1(2-3)-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 protein showed the best profile, binding more weakly than any of the other mutant proteins. The Mut4: Flt1(1-3_{$R\to N$})-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 be deleted from the primary sequence resulting in a decrease in charge interaction with extracellular matrix components.

(b.) Binding of Mut1: Flt1(1-3_{$\Delta B$})-Fc and Mut4: Flt1(1- $3_{R \to N}$ ) Fc in a BIACORETM-Based Assay.

Unmodified and acetylated Flt1 (1-3)-Fc and genetically modified Mut1; Flt1(1-3_{AB})-Fc and Mut4: Flt1(1-3_{B $\rightarrow N$})-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 40 chip (see BIACORETM Instruction 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, or 1.0 µg/ml), purified acetylated Flt1(1-3)-Fc (at (0.25, 0.5, or 1.0 µg/ml), COS cell supernatant containing Mut1: Flt1 (1-3 $_{\Delta B}$ )-Fc. (at approximately (0.25, 0.5, or 1.0 µg/ml), or COS cell supernatant containing Mut4: Flt1(1-3_{$R \rightarrow N$})-Fc (at approximately (0.25, 0.5, or 1.0 µg/ml) were passed over the Flt1(1-3)-Fc-coated chip. As shown in FIG. 17, at the sub-stoichiometric ratio  $(0.25 \ \mu g/ml \ Flt1(1-$ 3)-Fc of unmodified, acetylated or genetically modified samples vs. 01. µg/ml VEGF), there is insufficient Flt1(1-3)-Fc protein to block binding of VEGF to the Flt1(1-3)-Fc immobilized on the BIACORE™ chip. At 0.5 µg/ml of 55 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 BIA-CORE™ chip At 1.0 µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, which is approximately a 10:1 stoichiometric ratio, the Flt1(1-3)-Fc proteins are able to block binding of VEGF to the BIA-CORE™ chip, but they are not equivalent. Unmodified, acetylated, and Mut1: Flt1(1-3 $_{\Delta B}$ )-Fc are essentially equal in their ability to block VEGF binding, whereas Mut4: Flt1(1- $3_{R \to N}$ )-Fc is somewhat less efficient at blocking binding. These results confirm the hypothesis that it is possible to reduce the non-specific binding of a positively charged 20

molecule by genetically removing a linear sequence of predominantly negatively charged amino acids.

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

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

#### Example 16

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

In vivo experiments were designed to assess the pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-25  $3_{AB}$ )-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- $3_{\Delta B}$ )-Fc proteins (4 mice each). These mice were tail bled at 1, 2, 4, 30 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 35 FIG. 20, the Cmax for these reagents was as follows: Unmodified Flt1(1-3)-Fc-0.15 µg/ml; 40 fold molar excess acetylated Flt1(1-3)-Fc—1.5  $\mu$ g/ml; and Mut1: Flt1(1-3_{$\Delta B$})-Fc-0.7 µg/ml.

#### Example 17

#### Modified Flt1 Receptor Vector Construction

The rationale for constructing modified versions of the 45 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 50 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 net charge of +11) is not essential for binding, but confers higher affinity for VEGF than the second Ig domain, so instead of deleting it entirely, it was replaced with the equivalent domains of the Flt1 receptor relatives Flk1 (also known as VEGFR2) and Flt4 (also known as VEGFR3). These chimeric molecules (denoted R1R2 (Flt1.D2.Flk1D3.Fc $\Delta$ C1(a) and VEGFR1R2-Fc $\Delta$ C1 (a) and R1R3 (Flt1D2.VEGFR3D3-Fc $\Delta$ C1(a) and VEGFR₁R3-Fc $\Delta$ C1(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 (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 endothelial cells as described infra.

(a) Construction of the Expression Plasmid  $pFlt1D2.Flk1D3.Fc\Delta C1(a)$ 

Expression plasmids pMT21.Flt1(1-3).Fc (6519 bp) and pMT21.Flk-1(1-3).Fc (5230 bp) are plasmids that encode ampicillin resistance and Fc-tagged versions of Ig domains 1-3 of human Flt1 and human Flk1, respectively. These plasmids were used to construct a DNA fragment consisting of a fusion of Ig domain 2 of Flt1 with Ig domain 3 of Flk1, using PCR amplification of the respective Ig domains for 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
```

```
(5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3')
```

```
3': Flt1D2-Flk1D3.as
(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 (corresponding to amino acids 27-33 of FIG. **21A-21**C). 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 the fusion point defined as TIID of Flt1 (corresponding to amino acids 123-126 of FIG. **21A-21**C) and continuing into VVLS

(corresponding to amino acids 127-130 of FIG. **21A-21**C) of Flk1.

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

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 55 the end of Flk1 Ig domain 3, defined by the amino acids VRVHEK (corresponding-to amino acids 223-228 of FIG. **21A-21**C), followed by a bridging sequence that includes a recognition sequence for the restriction enzyme SrfI, and encodes the amino acids GPG. The bridging sequence 60 corresponds to amino acids 229-231 of FIG. **21A-21**C).

After a round of PCR amplification to produce the individual domains, the products were combined in a tube and subjected to a further round of PCR with the primers bsp/flt1D2 and Flk1D3/apa/srf.as (described supra) to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 614 bp fragment was subcloned into the BspEI

^{5&#}x27;: Flt1D2-Flk1D3.s

^{(5&#}x27;-ACAATCATAGATGTGGTTCTGAGTCCGTCTCATGG-3')

^{3&#}x27;: Flk1D3/apa/srf.as

^{(5&#}x27;-GATAATGCCCGGGCCCTTTTCATGGACCCTGACAAATG-3')

30

to SrfI restriction sites of the vector pMT21/ $\Delta$ B2.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 5 and the resulting 702 bp fragment was transferred into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)B2-Fc\DeltaC1(a) to produce the plasmid pFlt1D2.Flk1D3.Fc\DeltaC1 (a). The complete DNA and deduced amino acid sequences of the Flt1D2.Flk1D3.Fc\DeltaC1(a) chimeric molecule is set 10 forth in FIG. **21A-21**C.

(b) Construction of the Expression Plasmid  $pFlt1D2VEGFR3D3Fc\DeltaC1(a)$ 

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

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3')

3': Flt1D2.VEGFR3D3.as (TTCCTGGGCAACAGCTGGATATCTATGATTGTATTGGT)

The 5' amplification primer encodes a BspEI restriction site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (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 of Flt1 (corresponding to amino acids 123-126 of FIG. **22A-22C**) and continuing into IQLL 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 (ATCCAGCTGTTGCCCAGGAAGTCGCTGGAGCTGCTGGTA)

3': R3D3.as (ATTTTCATGCACAATGACCTCGGTGCTCTCCCGAAATCG)

Both the 5' and 3' amplification primers match the ⁵⁰ 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 ⁵⁵ GPG bridge (see below). The amplification primers were as follows:

5': Flt1D2.VEGFR3D3.s (TCATAGATATCCAGCTGTTGCCCAGGAAGTCGCTGGAG)

3': VEGFR3D3/srf.as (GATAATGCCCGGGCCATTTTCATGCACAATGACCTCGGT)

The 5' amplification primer encodes the 3' end of Flt1 Ig 65 domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplifi-

cation primer encodes the 3' end of VEGFR3 Ig domain 3, defined by the amino acids VIVHEN (corresponding to amino acids 221-226 of FIG. **22**A-**22**C), followed by a bridging sequence that includes a recognition sequence for SrfI, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 227-229 of FIG. **22**A-**22**C.

After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain 3) of PCR to produce the individual Ig domains, the PCR products were combined in a tube and subjected to a further round of PCR amplification with the amplification primers bsp/flt1D2 and VEGFR3D3/srf.as described supra, to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 625 bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/Flt1AB2.Fc (described supra), to create the plasmid pMT21/Flt1D2.VEGFR3D3.Fc. The sequence of the Flt1D2-VEGFR3D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes. EcoRI and SrfI and the resulting 693 bp fragment was subcloned into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3) $\Delta$ B2-Fc $\Delta$ C1(a) to produce plasmid the designated complete 25 pFlt1D2.VEGFR3D3.Fc $\Delta$ C1(a). The DNA deduced sequence acid of the amino Flt1D2.VEGFR3D3.Fc\DeltaC1(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) 35 were rehydrated with warm DME supplemented with glutamine (2 mM), 100 U penicillin, 100 U streptomycin, and 10% BCS for at least 1 hr. before adding samples. The plates were then incubated for 1 hr. at room temperature with varying concentrations of Flt1D2.Flk1D3.Fc\DeltaC1(a) and Flt1D2.VEGFR3D3.Fc∆C1(a) starting at 10 nM with subsequent 2-fold dilutions in PBS plus 10% BCS. The plates were then washed 3 times with PBS plus 0.1% Triton-X and incubated with alkaline phosphatase-conjugated anti-human Fc antibody (Promega, 1:4000 in PBS plus 10% BCS) for 1 hr. at room temperature. The plates were then washed 4 times with PBS 0.1% Triton-X and alkaline phosphatase buffer/pNPP solution (Sigma) was added for color development. Plates were read at I=405-570 nm. The results of this experiment are 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.FcΔC1(a) in CHO-K1 (E1A) Cells

A large scale (2 L) culture of *E. coli* DH1B cells carrying
the pFlt1D2.Flk1D3.FcΔC1(a) plasmid described supra in Example 17(a) 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 enzymes EcoRI plus Notl and Asel. 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 5 cells at a density of  $4 \times 10^6$  cells/plate. Plating media was Gibco Ham's F-12 supplemented with 10% HYCLONE™ Fetal Bovine Serum (EBS), 100 U penicillin/100 U streptomycin and glutamine (2 mM). The following day each plate of cells was transfected with 6 µg of the 10 pFlt1D2.Flk1D3.FcAC1(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 incu-15 bated at 37° C. in a 5% CO₂ incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II supplemented with glutamine (2 mM) and 1 mM sodium butyrate) was added. The plates were incubated at 37° C. for 3 days. 20 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 1L bottles and purification of the expressed protein was performed as described infra. 25

#### Example 20

## Construction pVEGFR1R2-Fc $\Delta$ 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. **24A-24**C) between Flt1d2-Flk1d3-Fc $\Delta$ C1(a) amino acids 26 and 27 of 35 FIG. **21A-21**C (GG) and removal of DNA encoding amino acids GPG corresponding to amino acids 229-231 of Figure. The SDT amino acid sequence is native to the Flt1 receptor and was added back in to decrease the likelihood of heterogeneous N-terminal processing. The GPG (bridging 40 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-24**C.

#### Example 21

## Cell Culture Process Used to Produce Modified Flt1 Receptors

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

The process for production of Flt1D2.Flk1D3.Fc $\Delta$ C1(a) protein using the expression plasmid pFlt1D2.Flk1D3.Fc $\Delta$ C1(a) described supra in Example 1 involves suspension culture of recombinant Chinese hamster ovary (CHO K1/E1A) cells which constitutively express the protein product. The cells are grown in bioreactors and the protein product is isolated and purified by affinity and size exclusion chromatography. The process is provided in ⁶⁰ greater detail below.

#### Cell Expansion

Two confluent T-225 cm² flasks containing the Flt1D2.Flk1D3.Fc $\Delta$ C1(a) expressing cell line were 65 expanded by passaging cells into eight T-225 cm² flasks in medium (GMEM+10% serum, GIBCO) and incubated at

 $37^{\circ}$  C. and 5% CO₂. When the flasks approached confluence (approximately 3 to 4 days) the cells were detached using trypsin. Fresh medium was added to protect the cells from further exposure to the trypsin. The cells were centrifuged and resuspended in fresh medium then transferred to eight 850 cm² roller bottles and incubated at  $37^{\circ}$  C. and 5% CO₂ 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 5L bioreactor (New Brunswick Celligen Plus) where the cells are grown in 3.5L of suspension 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  $4 \times 10^6$  cells/mL was reached the cells were transferred to a 40L 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.

(b) Cell Culture Process Used to Produce  $_{30}$  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

(a) Harvest and Purification of Flt1D2.Flk1D3.Fc $\Delta$ C1(a)

40 The 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.
45 Approximately 40L of harvest filtrate was then loaded onto a 400 mL column containing Protein A SEPHAROSETM resin (Amersham Pharmacia). After loading the resin was washed with buffer containing 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.2 to remove any unbound 50 contaminating proteins. Flt1 D2.Flk1D3.FcΔC1(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° C.

Several frozen lots of Flt1D2.Flk1D3.Fc $\Delta$ C1(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 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.Fc $\Delta$ C1(a) dimer were pooled, sterile filtered through a 0.22 micron filter, aliquoted and frozen. (b) Harvest and Purification of Flt1D2.VEGFR3D3.Fc $\Delta$ C1 (a)

The same methodologies as described supra for Flt1D2.Flk1D3.Fc $\Delta$ C1(a) were used to harvest and purify Flt1D2.VEGFR3D3.Fc $\Delta$ C1(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, Flk1 and Flt4(VEGFR3) were prepared and were preincubated for 1 hr. at room temperature with varying amounts of the modified Flt1 receptors Flt1(1-3)-Fc, 20 Flt1(1-3)-Fc (A40), Flt1D2Flk1D3.FcΔC1(a) and Flt1 D2VEGFR3D3.Fc $\Delta$ C1(a) in serum-free DME-high glucose media containing 0.1% BSA. Cells were challenged for minutes with the samples prepared above ±VEGF165, followed by whole cell lysis using complete lysis buffer. Cell 25 lysates were immunoprecipitated with an antibody directed against the C-terminus of VEGFR2 receptor. The immunoprecipitated lysates were loaded onto 4-12% SDS-PAGE Novex gel and then transferred to PVDF membrane using standard transfer methodologies. Detection of phosphorylated VEGFR2 was done by immunoblotting with the antiphospho Tyrosine mAb called 4G10 (UBI) and developed using ECL-reagent (Amersham).

FIGS. 25A-25C and 26A-26B show the results of this experiment. FIG. 25A-25C reveals that detection by Western  35 blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are phosphorylated to varying levels depending on which modified Flt1 receptor is used during the preincuba-40 tions 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.Fc∆C1(a) there is complete blockage of receptor stimulation by these three modified Flt1 receptors as compared to control media challenge. In contrast, tran- $_{45}$ sient Flt1D2VEGFR3D3.Fc $\Delta$ C1(a) does not show significant blockage at this molar excess, as compared with VEGF positive control challenge. Similar results are seen in FIG. 25B, where the modified Flt receptors are in a 3-fold molar excess to VEGF165 ligand. In FIG. 25C, where the modified 50 Flt1 receptors are in a 6-fold molar excess to VEGF165 ligand, transient Flt1D2VEGFR3D3.Fc∆C1(a) can now be shown to be partially blocking VEGF165-induced stimulation of cell-surface receptors.

In FIG. **26A-26**B, detection by Western blot of tyrosine ⁵⁵ phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are not phosphorylated by challenge samples which have VEGF165 preincubated with 1 and 2 fold molar excess (FIG. **26**A) or 3 and 4 fold molar excess (FIG. **26**B) of either transient Flt1D2Flk1D3.Fc $\Delta$ C1(a), stable Flt1D2Flk1D3.Fc $\Delta$ C1(a), or transient VEGFR1R2-Fc $\Delta$ C1(a). At all modified Flt1 receptor concentrations tested there is complete binding of VEGF165 ligand during the preincubation, resulting in no 65 detectable stimulation of cell surface receptors by unbound VEGF1-65 as compared to control media challenge.

## Example 24

#### Cell Proliferation Bioassay

5 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 intra-10 cellular kinase domain was used rather than the native VEGFR2Flk1) intracellular kinase domain is that the intracellular kinase domain of VEGFR2Flk1) does not cause a strong proliferative response when stimulated by VEGF165 in these cells. It is known that MG87 cells containing full 15 length TrkB receptor give a robust proliferative response when stimulated with BDNF, so the TrkB intracellular kinase domain of VEGFR2Flk1) to take advantage of this proliferative response capability.

 $5 \times 10^3$  cells/well were plated in a 96 well plate and allowed to settle for 2 hrs at 37° C. The following modified Flt receptors Flt1(1-3)-Fc, Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and Flt1 D2.VEGFR3D3.FcΔC1(a), plus an irrelevant receptor termed 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 plates were incubated for 72 hrs at 37° C. and then MTS (Owen's reagent, Promega) added and the plates were incubated for an additional for 4 hrs. Finally, the plates were read on a spectrophotometer at 450/570 nm. The results of this experiment are shown in FIG. 27. The control receptor Tie2-Fc does not block VEGF165-induced cell proliferation at any concentration whereas Flt1D2.Flk1D3.Fc $\Delta$ C1(a) blocks 1.56nM VEGF165 with a half maximal dose of 0.8 nM. Flt1(1-3)-Fc and Flt1D2.VEGFR3D3.Fc\DeltaC1(a) are less effective in blocking VEGF165 in this assay with a half maximal dose of ~2nM. 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

## (a) BIACORE[™] 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 Flt1D2Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a) surfaces or measuring concentration of VEGF165 needed to completely prevent binding of Flt1D2Flk1D3.FcC1(a) or VEGFR1R2-Fc $\Delta$ C1(a) to VEGF BlACORETM chip surface.

Modified Flt receptors Flt1D2Flk1D3.Fc $\Delta$ C1(a) and VEGFR1R2-Fc $\Delta$ C1(a), were captured with an anti-Fc specific antibody that was first immobilized on a BIACORETM 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 overthe Flt1D2Flk1D3.Fc $\Delta$ C1(a) and VEGFR1R2-Fc $\Delta$ C1 (a) surfaces at 10 µl/mm for one hour. A real-time binding signal was recorded and saturation binding was calculated as a molar ratio of bound VEGF165 to the immobilized
Flt1D2Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a), using the conversion factor of 1000 RU equivalent to 1 ng/ml. The results indicated binding stoichiometry of one VEGF165 dimeric molecule per one Flt1 D2Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a) molecule (FIG. **28**).

In solution, Flt1D2Flk1D3.Fc∆C1(a) or VEGFR1R2- $Fc\Delta C1(a)$  at a concentration of 1 nM (estimated to be 1000 times higher than the KD of the Flt1 D2Flk1 D3.Fc $\Delta$ C1(a) or VEGFR1 R2-Fc\DeltaC1(a)NEGF165 interaction) were mixed with varied concentrations of VEGF165. After one 10 incubation. concentrations of the free hour Flt1D2Flk1D3.Fc $\Delta$ C1(a) in solution were measured as a binding signal to an amine-coupled VEGF165 surface. A calibration curve was used to convert the Flt1D2Flk1D3.Fc∆C1(a) BIACORE™ binding signal to its 15 molar concentration. The data showed that the addition of 1 nM VEGF165 into the Flt1D2Flk1D3.Fc\DeltaC1(a) solution completely blocked Flt1D2Flk1D3.Fc $\Delta$ C1(a) binding to the VEGF165 surface. This result suggested the binding stoichiometry of one VEGF165 molecule per one 20 Flt1D2Flk1D3.Fc $\Delta$ C1(a) molecule (FIG. **29** and FIG. **30**). When the concentration of Flt1D2Flk1D3.Fc $\Delta$ C1(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 25 (a). The magnitude of the slope, very close to negative one, was indicative that one molecule of VEGF165 bound to one molecule of either Flt1D2Flk1D3.Fc\DeltaC1(a) or VEGFR1 R2-Fc $\Delta$ C1(a).

#### (b) 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 SUPEROSETM 6 size exclusion chromatography column. The receptor-ligand complex was then 35 incubated in a buffer containing 6M guanidine hydrochloride in order to dissociate it into its component proteins. Flt1D2Flk1D3.Fc\DeltaC1(a) was separated from VEGF165 using SUPEROSE™ 6 size exclusion chromatography column run in 6M guanidium chloride. In order to determine 40 complex stoichiometry, several injections of Flt1D2Flk1D3.FcΔC1(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.Fc\DeltaC1(a)VEGF complex. Quantification of the Flt1D2Flk1D3.Fc $\Delta$ C1(a)/ VEGF complex composition was based on the calibration curves. The results of this experiment are set forth in FIG. 28. which shows the ratio of VEGF165 to Flt1D2Flk1D3.Fc $\Delta$ C1(a) in a complex to be 1:1.

#### Example 26

#### Determination of the Binding Stoichiometry of Flt1D2Flk1D3.Fc∆C1(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=0.9 mg/ml) in molar ratio of 3:1 (VEGF165: Flt1D2.Flk1D3.FcΔC1(a)) and incubated overnight at 4° C.

(a) 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  $\mu$ l/min. at room temperature. The results of this SEC are shown in FIG. **31**. Peak #1 represents the complex and peak #2 represents unbound VEGF165. Fractions eluted between 1.1 and 1.2 ml were combined and guanidinium hydrochloride (GuHCI)was added to a final concentration 4.5M to dissociate the complex.

(b) Size Exclusion Chromatography (SEC) Under Dissociative Conditions

To separate the components of the receptor-ligand complex and to determine their molar ratio, 50  $\mu$ l of dissociated complex as described supra was loaded onto a SUPER-OSETM 12 PC 3.2/30 equilibrated in 6M GuHCl and eluted with the same solution at a flow rate 40  $\mu$ l/min. at room temperature. The results of this SEC are shown in FIG. 32.

(c) Calculation of Flt1D2Flk1D3.FcΔC1(a):VEGF165 Complex Stoichiometry

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

#### Example 27

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

#### ⁴⁵ Complex Preparation

VEGF165 was mixed with CHO transiently expressed Flt1D2.Flk1D3.Fc $\Delta$ C1(a) protein in molar ratio of 3:1 (VEGF165:Flt1D2Flk1D3.Fc $\Delta$ C1(a)) and incubated overnight at 4° C.

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

Size exclusion chromatography column with a MiniDawn on-line light scattering detector (Wyatt Technology, Santa
55 Barbara, Calif.) and refractive index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine the molecular weight (MW) of the receptor-ligand complex. Samples were injected onto a SUPEROSE™ 12 HR 10/30 column (Pharmacia) equilibrated in PBS buffer and eluted with the same
60 buffer at flow rate 0.5 ml/mm. at room temperature. As shown in FIG. 33, the elution profile shows two peaks. Peak #1 represents the receptor-ligand complex and peak #2 represents the unbound VEGF165. MW was calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.FcΔC1(a)/VEGF-165

complex at the peak position is 157 300 (FIG. **33**), the MW of VEGF165 at the peak position is .44 390 (FIG. **34**) and the MW of R1R2 at the peak is 113 300 (FIG. **35**).

These data indicated that the stoichiometry of the Flt1D2Flk1D3.Fc $\Delta$ C1(a)NEGF complex is 1:1 as its corresponds to the sum of molecular weights for Flt1D2Flk1D3.Fc $\Delta$ C1(a) and VEGF165. Importantly, this method conclusively proved that the Flt1D2Flk1D3.Fc $\Delta$ C1(a) (a)/VEGF165 complex was indeed composed of only one molecule of VEGF165 ligand and only one molecule of the 10 Fc $\Delta$ C1(a) (a). FIG. **37**. The T_{max} for Flt the T_{max} for the transient VEC C_{max} for Flt1(1-3)-Fc (A4 (Flt1D2.Flk1D3.Fc $\Delta$ C1(a) (a)/VEGF165 ligand and only one molecule of the 10 Fc $\Delta$ C1(a) was 30 µg/ml.

#### Example 28

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

The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.Fc $\Delta$ C1(a) were determined by a peptide mapping method. In this method, the protein was first cleaved with trypsin. Tryptic fragments were analyzed and ²⁰ 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 ²⁵ help identify fragments with N-linked glycosylation sites. The results are summarized in the accompanying FIG. **36**.

are a total of There ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to-the Fc region. Cys127 has been confirmed to be disulfide bonded to 30 Cys76. Cys121 is confirmed to be disulfide bonded to Cys 182. 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 35 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. Cys 352 is confirmed to be disulfide bonded to Cys410.

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

#### Example 29

#### Pharmacokinetic Analysis of Modified Flt Receptors

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

Balb/c mice (25-30 g) were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.Fc $\Delta$ C1(a), CHO stably expressed 60 Flt1D2.Flk1D3.Fc $\Delta$ C1(a), and CHO transiently expressed VEGFR1R2-Fc $\Delta$ C1(a). The mice were tail bled at 1, 2, 4, 6, 24 hrs, 2 days, 3 days and 6 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a). 65 The ELISA involves coating an ELISA plate with VEGF165, binding the detect Flt1(1-3)-Fc (A40),

Flt1D2.Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(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.Fc $\Delta$ C1 (a) and the transient VEGFR1R2-Fc $\Delta$ C1(a) was 24 hrs. The C_{max} for Flt1(1-3)-Fc (A40) was 8 µg/ml. For both transients (Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and VEGFR1R2-Fc $\Delta$ C1(a)) the C_{max} was 18 µg/ml and the C_{max} for the stable VEGFR1R2-Fc $\Delta$ C1(a) was 30 µg/ml.

(b) Pharmacokinetic Analysis of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and Flt1 D2.VEGFR3D3.Fc $\Delta$ C1 (a)

Balb/c mice (25-30 g) were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and CHO transiently expressed Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a). The mice were tail bled at 1, 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1 D2.VEGFR3D3.FcΔC1 (a). The ELISA involves coating an ELISA plate with 165, binding the Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) or Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. Flt1(1-3)-Fc (A40) could no longer be detected in the serum after day 5 whereas Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a) were detectable for 15 days or more. The results of this experiment are shown in FIG. 38.

#### Example 30

#### Evaluation of the Ability of Flt1D2.Flk1D3.Fc∆C1(a) to Inhibit Tumor Growth In Vivo

To evaluate the ability of Flt1D2.Flk1D3.Fc $\Delta$ C1(a) to inhibit tumor growth in vivo a model in which tumor cell suspensions are implanted subcutaneously on the right flank of male severe combined immunodeficiency (SCID) mice was employed. Two cell lines, the human HT-1080 fibrosarcoma cell line (ATCC accession no. CCL-121) and the rat C6 glioma cell line (ATCC accession no. CCL-107), each of which exhibit distinctly different morphologies and growth characteristics, were used in the assay. The first dose of Flt1D2.Flk1D3.Fc\DeltaC1(a) (at 25 mg/Kg or as indicated in FIGS. 39 and 40) was given on the day of tumor implantation. Animals subsequently received subcutaneous injections of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or 50 vehicle either every other day (EOD) or two times per week (2×/wk) for a period of 2 weeks. After 2 weeks, animals were perfused with fixative, tumors were removed and samples were blinded. Tumor volume was determined by measuring the length and width of visible subcutaneous tumors. Both of Flt1(1-3)-Fc (A40) and Flt1D2.Flk1D3.Fc $\Delta$ C1(a) significantly reduced the growth of tumors formed by HT-1080 and C6 cells. The results of these experiments are shown in FIG. 39 and FIG. 40.

#### Example 31

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

The stereotypic pattern of vascular remodeling which occur in the uterus and ovary over the course of the reproductive cycle has been well characterized, making these tissues particularly well suited to the study of mechanisms which regulate angiogenesis, vascular remodeling and vascular regression. Indeed, in situ hybridization studies in the reproductive tissues provided the first clear evidence that VEGF acts as a mediator of physiological angiogenesis in 5 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 10 vessel growth and/or vascular dysfunction have been found to characterize many pathological conditions which affect these organs. Furthermore, these pathogenic vascular abnormalities are thought to be caused or perpetuated by the dysregulated expression of one or more angiogenic or anti-15 angiogenic factors, most prominently VEGF.

For example, abnormal angiogenesis is characteristic of polycystic ovary disease, endometriosis and endometrial carcinoma, and in each case VEGF is over expressed in the affected tissue (Kamat et al, 1995; Shifren et al, 1996; Guidi 20 et al, 1996; Donnez et al, 1998). Overexpression of VEGF is also thought to play a pathogenic role in the establishment of systemic vascular hyperpermeability in ovarian hyperstimulation syndrome (McClure et al, 1994; Levin, et al, 1998) and preeclampsia (Baker et al, 1995; Sharkey et al, 25 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 30 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 35 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 (Sh- 40 weiki 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 biologi- 45 cal actions of VEGF may prove to be useful as contraceptive agents (by preventing implantation), or as an abortifacients in the early stages of gestation. The latter application might find particular use as a non-surgical intervention for the termination of ectopic pregnancies. 50

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 tropho-55 blast 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 vas-65 culature, but that at least in some tumors of reproductive origin VEGF may subserve an autocrine role, directly sup-

porting the survival and proliferation of the tumor cells. Thus agents which block the actions of VEGF may have particularly beneficial applications to the treatment of tumors of reproductive origin.

### Methods and Results

(a) Assessment of VEGF-Induced Uterine Hyperpermeability

Pregnant mare'serum gonadotrophin (PMSG) was injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats. This results in a surge of estradiol after 2 days which in turn causes an induction of VEGF in the uterus. It is reported that this induction results in hyperpermeability 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) and Flt1 D2.VEGFR3D3.Fc $\Delta$ C1 (a) at 25 mg/kg at 1 hr. after PMSG injection results in about a 50% inhibition of the increase in uterine wet weight. Increasing the dose of modified Flt receptor does not further reduce the increase in wet weight suggesting that there is a VEGF-independent component to this model. The results of this experiment are shown in FIG. 41.

(a) 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 female rats. This results in a fully functioning corpus luteum containing a dense network of blood vessels after 4 days that allows for the secretion of progesterone into the blood stream in order to prepare the uterus for implantation. The induction of angiogenesis in the corpus luteum requires VEGF; therefore, blocking VEGF would result in a lack of new blood vessels and thus a lack of progesterone secreted into the blood stream. In this in vivo model, resting levels of progesterone are about 5 ng/ml and this can be induced to a level of 25-40 ng/ml after PMSG. Subcutaneous injection of Flt1(1-3)-Fc (A40) or Flt1D2.Flk1D3.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.

#### 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 Tmax 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

10 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.Fc $\Delta$ C1(a), transiently expressed Flt1D2VEFGFR3D3-Fc $\Delta$ C1(a), Flt1-(1- ${}^{3}_{MAS}$ )-Fc, Flt1(1- ${}^{3}_{R\rightarrow C}$ )-Fc and Tie2-Fc. Flt1(1- 5  ${}^{3}_{MAS}$ )-Fc is a modified version of Flt1(1-3)-Fc in which the highly basic amino acid sequence KNKRASVRRR is replaced by NASVNGSR, resulting in the incorporation of two new glycosylation sites and a net reduction of five positive charges, both with the purpose of reducing the 10 unfavorable effects of this sequence on PK. Flt1(1- ${}^{3}_{R\rightarrow C}$ )-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→KNKCASVRRR) to allow for pegylation at that residue, which could then shield the basic region from exerting its unfavorable effects on PK. After incubation the solution was transferred to a plate containing a capture antibody for VEGF165 (R&D). The amount of free VEGF165 was then determined using an antibody to report free VEGF165. This showed that the modified Flt1 receptor variant with the highest affinity for VEGF165 (determined as the lowest amount of free VEGF165) was Flt1D2Flk1D3.Fc $\Delta$ C1(a), followed by Flt1 (1-3)-Fc and Flt1(1-3)-Fc (A40) and then by Flt1(1-3_{*R*→*C*})-Fc, Flt1(1-3_{*NAS*})-Fc and Flt1D2VEFGFR3D3-Fc $\Delta$ C1(a). Tie2Fc has no affinity for VEGF165.

<160	)> NU	JMBEH	S OF	SEQ	ID N	10S :	38									
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<400	)> SH	EQUEI	ICE :	1												
atg Met 1	gtc Val	agc Ser	tac Tyr	tgg Trp 5	gac Asp	acc Thr	ggg Gly	gtc Val	ctg Leu 10	ctg Leu	tgc Cys	gcg Ala	ctg Leu	ctc Leu 15	agc Ser	48
tgt Cys	ctg Leu	ctt Leu	ctc Leu 20	aca Thr	gga Gly	tct Ser	agt Ser	tca Ser 25	ggt Gly	tca Ser	aaa Lys	tta Leu	aaa Lys 30	gat Asp	cct Pro	96
gaa Glu	ctg Leu	agt Ser 35	tta Leu	aaa Lys	ggc Gly	acc Thr	cag Gln 40	cac His	atc Ile	atg Met	caa Gln	gca Ala 45	ggc Gly	cag Gln	aca Thr	144
ctg Leu	cat His 50	ctc Leu	caa Gln	tgc Cys	agg Arg	999 Gly 55	gaa Glu	gca Ala	gcc Ala	cat His	aaa Lys 60	tgg Trp	tct Ser	ttg Leu	cct Pro	192
gaa Glu 65	atg Met	gtg Val	agt Ser	aag Lys	gaa Glu 70	agc Ser	gaa Glu	agg Arg	ctg Leu	agc Ser 75	ata Ile	act Thr	aaa Lys	tct Ser	gcc Ala 80	240
tgt Cys	gga Gly	aga Arg	aat Asn	ggc Gly 85	aaa Lys	caa Gln	ttc Phe	tgc Cys	agt Ser 90	act Thr	tta Leu	acc Thr	ttg Leu	aac Asn 95	aca Thr	288
gct Ala	caa Gln	gca Ala	aac Asn 100	cac His	act Thr	ggc Gly	ttc Phe	tac Tyr 105	agc Ser	tgc Cys	aaa Lys	tat Tyr	cta Leu 110	gct Ala	gta Val	336
cct Pro	act Thr	tca Ser 115	aag Lys	aag Lys	aag Lys	gaa Glu	aca Thr 120	gaa Glu	tct Ser	gca Ala	atc Ile	tat Tyr 125	ata Ile	ttt Phe	att Ile	384
agt Ser	gat Asp 130	aca Thr	ggt Gly	aga Arg	cct Pro	ttc Phe 135	gta Val	gag Glu	atg Met	tac Tyr	agt Ser 140	gaa Glu	atc Ile	ccc Pro	gaa Glu	432
att Ile 145	ata Ile	cac His	atg Met	act Thr	gaa Glu 150	gga Gly	agg Arg	gag Glu	ctc Leu	gtc Val 155	att Ile	ccc Pro	tgc Cys	cgg Arg	gtt Val 160	480
acg Thr	tca Ser	cct Pro	aac Asn	atc Ile 165	act Thr	gtt Val	act Thr	tta Leu	aaa Lys 170	aag Lys	ttt Phe	cca Pro	ctt Leu	gac Asp 175	act Thr	528
ttg Leu	atc Ile	cct Pro	gat Asp 180	gga Gly	aaa Lys	cgc Arg	ata Ile	atc Ile 185	tgg Trp	gac Asp	agt Ser	aga Arg	aag Lys 190	ggc Gly	ttc Phe	576

SEQUENCE LISTING

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41

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gca Ala	aca Thr 210	gtc Val	aat Asn	с1 ^у ааа	cat His	ttg Leu 215	tat Tyr	aag Lys	aca Thr	aac Asn	tat Tyr 220	ctc Leu	aca Thr	cat His	cga Arg	672
caa Gln 225	acc Thr	aat Asn	aca Thr	atc Ile	ata Ile 230	gat Asp	gtc Val	caa Gln	ata Ile	agc Ser 235	aca Thr	cca Pro	cgc Arg	cca Pro	gtc Val 240	720
aaa Lys	tta Leu	ctt Leu	aga Arg	ggc Gly 245	cat His	act Thr	ctt Leu	gtc Val	ctc Leu 250	aat Asn	tgt Cys	act Thr	gct Ala	acc Thr 255	act Thr	768
ccc Pro	ttg Leu	aac Asn	acg Thr 260	aga Arg	gtt Val	caa Gln	atg Met	acc Thr 265	tgg Trp	agt Ser	tac Tyr	cct Pro	gat Asp 270	gaa Glu	aaa Lys	816
aat Asn	aag Lys	aga Arg 275	gct Ala	tcc Ser	gta Val	agg Arg	cga Arg 280	cga Arg	att Ile	gac Asp	caa Gln	agc Ser 285	aat Asn	tcc Ser	cat His	864
gcc Ala	aac Asn 290	ata Ile	ttc Phe	tac Tyr	agt Ser	gtt Val 295	ctt Leu	act Thr	att Ile	gac Asp	aaa Lys 300	atg Met	cag Gln	aac Asn	aaa Lys	912
gac Asp 305	aaa Lys	gga Gly	ctt Leu	tat Tyr	act Thr 310	tgt Cys	cgt Arg	gta Val	agg Arg	agt Ser 315	gga Gly	cca Pro	tca Ser	ttc Phe	aaa Lys 320	960
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tgt Cys	ctg Leu	ctt Leu	ctc Leu 20	aca Thr	gga Gly	tct Ser	agt Ser	tca Ser 25	ggt Gly	tca Ser	aaa Lys	tta Leu	aaa Lys 30	gat Asp	cct Pro	96
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 Asn Gly Pro Gly Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro

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 Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys

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95

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We claim:

1. A fusion protein, consisting of

- (a) a vascular endothelial growth factor (VEGF) receptor component having immunoglobulin-like (Ig) domains consisting of an Ig domain 2 of a first VEGF receptor 5 human Flt1 and Ig domain 3 of a second VEGF receptor human Flk1; and
- (b) a multimerizing component, wherein the fusion protein binds VEGF.

**2**. The fusion protein of claim **1**, wherein the first VEGF 10 receptor component is upstream of the second VEGF receptor component.

**3**. The fusion protein of claim **1**, wherein the first VEGF receptor component is downstream of the second VEGF receptor component.

**4**. The fusion protein of claim **1**, wherein the multimerizing component comprises an immunoglobulin domain.

**5**. The fusion protein of claim **4**, wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, and the heavy chain of IgG.

**6**. A fusion protein encoded by a nucleic acid sequence selected from:

(a) SEQ ID NO:15; and

(b) nucleic acid sequences which, as a result of the degeneracy of the genetic code, differ from the nucleic acid sequence of SEQ ID NO:15.

10 7. A fusion protein consisting of immunoglobulin-like (Ig) domain 2 of a first vascular endothelial growth factor (VEGF) receptor upstream of Ig domain 3 of a second VEGF receptor and a multimerizing component, wherein the fusion protein comprises the amino acid sequence SEQ ID 15 NO:16.

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