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(12) United States Patent

Papadopoulos et al.

(54) MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES AND METHODS OF USING THEREOF

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This patent is subject to a terminal disclaimer.

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- (51) Int. Cl.

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

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

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(57) ABSTRACT

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

3 Claims, 55 Drawing Sheets













Fig.6B.









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Fig.10B.

GGA AAA CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA CCT TTT GOG TAT TAG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys> 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> CTC ACA CAT OGA CAA ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC GAG TGT GTA GCT GTT TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val> AAA TTA CTT AGA GGC CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG TTT AAT GAA TCT CCG GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG TGA GGG AAC TTG TGC Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr> AGA GTT CAA ATG ACC TGG AGT TAC CCT GAT GAA AAA AAT AAG AGA GCT TCC GTA AGG CGA TCT CAA GTT TAC TGG ACC TCA ATG GGA CTA CTT TTT TTA TTC TCT CGA AGG CAT TCC GCT Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys Asn Lys Arg Ala Ser Val Arg Arg> 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 Arg Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys> ATG CAG AAC AAA GAC AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT GGA CCA TCA TTC AAA TAC GTC TTG TTT CTG TTT CCT GAA ATA TGA ACA GCA CAT TCC TCA CCT GGT AGT AAG TTT Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Fhe Lys> TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC GAG CCC AAA TCT TGT AGA CAA TTG TGG AGT CAC GTA TAT ATA CTA TTT CGT CCG GGC CCG CTC GGG TTT AGA ACA Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys>

GAC AAA ACT CAC ACA TGC CCA COG TGC CCA GCA CCT GAA CTC CTG GGG GGA COG TCA GTC CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val> Fig.10C.

TTO OTO TTO COO CEA AAA COO AAG GAC ACO OTO ATG ATC TOO CGG ACO COT GAG GTO ACA AAG GAG AAG GOG GET TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT Fhe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr> * TGC GTG GTG GTG GAC GTG ACC CAL GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC ACG CAC CAC CTG CAC TOG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG Cys Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Fhe Asn Trp Tyr Val Asp> GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC COG 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> CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG GCA CAC CAG TOG CAG GAG TOG CAG GAC GTG GTC CTG ACC GAC TTA COG TTC CTC ATG TTC Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys> TOC ANG GTC TOC AND ANA GCC CTC CCA GOC COC ATC GAG ANA AOC ATC TOC ANA GCC ANA ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys> * * * GGG CAS CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys> * AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT COG AAG ATA GGG TCG CTG TAG CGG CAC CTC Asn Gin Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu> * * * ÷ TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser>

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.

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Fig. 13A.

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## Fig.13B.

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CIC	ACA	CAT	CGA	CAA	ACC	ААТ	aca	ATC	ATA	GAT	GTC	CAA	ATA	AGC	ACA	CCA	CGC	CCA	GTC
GAG	TGT	GTA	GCT	GIT	ĨGG	TTA	TGT	TAG	TAT	CTA	CAG	GTT	TAT	TCG	TGT	GGT	GCG	GGT	CAG
Leu	Thr	His	Arg	Gln	Inr	Asn	Thr	Ile	Ile	Asp	Val	Gln	Ile	Ser	Thr	Pro	Arg	Pro	Val>
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		7.	10		•	740			750			70	50		•	770			780
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mmm		CPT	ALIA	GGC	CAT	ACT	CIT	GIC	CIC	AAT	TGT	ACT	GCT	ACC	ACT	ccc	TIG	AAC	ACG
Lin	Tan	GRA Tour	TUT	COG	GTA	IGA	GAA	CAG	GAG	TTA	ACA	TGA	CGA	IGG	TGA	GGG	AAC	TIG	TGC
Lys	나면다	ren	Arg	GIY	HIS	Thr	Leu	Val	Leu	Asn	Cys	Thr	Ala	Thr	Thr	Pro	Leu	Asn	Thr>
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Arg	Val	Gln	Met	Thr	Trans	Ser	- -	Son Dro	ben	C11	TIA		GPP	106	TTA	فاقلام .	GIA	فخال .	TIG
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ATA	TTC	TAC	AGT	GTT	CTT	ACT	ATT	GAC	АЛА	ATG	CAG	AAC	AAA	GAC	222	GCA	CIT	ጥልጥ	ACT
TAT	AAG	ATG	TCA	CAA	GAA	TGA	TAA	CIG	TTT	TAC	GTC	TTG	TTT	CIRC	- datata		GAN	מתע	TCA
Ile	Phe	Tyr	Ser	Val	Leu	Thr	Ile	Asp	Lys	Met	Gln	Asn	Lvs	ASD	LVS	Glv	Leu	Tvr	Thr>
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TGT	CGT	GTA	AGG	AGT	GGA	CCA	TCA	TTC	ААА	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>
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INF	201	Glue	Dro	CCG (21	CIC CIC	GGG	TTT	AGA	ACA	CIG	TTT	TGA	GTG	TGT	ACG	GGT	GGC	ACG	GGT
-33		OLY.	510	Grð	GIU	FIO	LYS	ser	Cys	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Сув	Pro>
		10	30		1	040			1050			10	60			070			
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GCA	CCT	GAA	CTC	CTG	GGG	GGA	006	TCA	GTC	יאדיני	CTC	<b>T</b> TC-	- COM	- «^^^	2	. ~~~	220	-	-
CGT	GGA	CIT	GAG	GAC	CCC	COT	000	201	C20		~~~		~~~~						
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Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	GAG Leu	AAG Phe	Pro	) Pro	LV8	GGG	IVs	Ast	o Thr>

### Fig.13C.

CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GAC GTG AGC CAC GAA GAC GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp> CCT GAG GTC AAG TTC AAC TEG TAC GTG GAC GEC 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> 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> 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> CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC GGG TAG CTC TIT 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> 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> 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> * 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> 

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.

		163	30		16	540		1	650			166	50		16	70	
	*		*	*		*		*	×		*		*	*		*	
GCT	CTG	CAC	AAC	CAC	TAC	ACG	CAG	AAG	AGC	CTC	TCC	CTG	TCT	CCG	GGT	ааа	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.

20 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 TOG ATG ACC CTG TOG CCC CAG GAC GAC ACG CGC GAC GAG TOG ACA GAC GAA GAG Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu-70 80 90 100 110 120 * 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> 140 130 150 160 170 180 * * 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> 190 200 210 220 230 240 * 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 GOG TAT TAG Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile> 250 260 270 280 290 300 * 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> 310 320 330 340 350 360 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> 390 370 420 380 400 410 * 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> 480 430 440 450 460 470 * 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> 500 490 510 520 530 540 ٠ * * 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>

## Fig.14B.

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GAA	TGA	AAT	CTG	TTT	TAC	GTC	TTG	TTT	CTG	TTT	ÇCT	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>
		61	10		e	520			630			64	lÓ		6	50			660
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GGA	CCA	TCA	TTC	AAA	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	CCCG
Gly	Pro	Ser	Phe	Lys	Ser	Val	Asn	Thr	Ser	Val	His	Ile	Tyr	Asp	Lys	Ala	Gly	Pro	Gly>
		61	70		(	580			690			7(	00			710			720
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GAG	ccc	АЛА	TCT	TGT	GAC	ааа	ACT	CAC	ACA	TGC	CCA	ccc	TGC	CCA	GCA	CCT	GAA	CTC	CTG
CIC	GGG	TTT	AGA	ACA	CTG	TTT	TGA	GTG	TGT	ACG	GGT	GGC	ACG	GGT	CGT	GGA	CTT	GAG	GAC
Glu	Pro	Lys	Ser	Суз	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Сув	Pro	Ala	Pro	Glu	Leu	Leu>
		73	30			74N			750			71	50			770			780
	<b>±</b> .		*	*		*		*	*		•		*	*		*		*	*
GGG	GGA	CCG	TCA	GTC	TTC	CTC	TIC	ccc	CCA	AAA	ccc	AAG	GAC	ACC	CTC	ATG	ATC	TCC	CGG
œ	CCT	GGC	AGT	CAG	AAG	GAG	AAG	GGG	GGT	TTT	GGG	TTC	CIG	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>
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	*		*	*		500 *		+	810		*	8.	20		i	530		•	840
200	COT	GAG	CTC	<b>BCB</b>	mac	CTTC	010	CTTC:	GNC.	one	200	<b>~</b> >~	~ ~ ~ ~	<b>C</b> NC	~~~	676	C-III-	280	anno.
TGG	GCI		CJC	TOT	100	CPC	CIC	CAC	CTC	C C C C C C C C C C C C C C C C C C C	TOC	CTC	CTTT				CIC	100	ANC N
Thr	Pro	Glu	Val	Thr	Суб	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe>
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11G	MUC The	MIG The	UAL Val		Clu	UAL:	CIC	UAC Vol	GIA	11A	وي ال	Tim	TGT	TIC	GGC	GCC	CIU	010	GIC
1011	тр	TÀT	vai	Asp	GIŶ	vai	Ģru	var	n12	ASII	Ala	рув	Int	гув	PIO	ΑĽĠ	Gru	GTU	
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m2.0		200	-	- -	~~~~	-	~~~		~~~~	~~~		~~~	~~~~	~~~~	~~~			-	***
200	AAC	moo	MCG	AC	CGT	GIG	GIC	AGC	GIC	CIC	ACC	GIC	CIG		فلقت	GAC	166	CIG	AAT
Tyr	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn>
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000	220	~~~	-	*	<b>~</b> ~~	*	~~~	- 	*			~~~	*	*		*	~~~		*
~~~	MAG	GAG	TAC	AAG	160	AAG	GIC	100	AAC	AAA	GCC	CIC	CCA	GCC	CCC	ATC	GAG	AAA	
CLG Clu	Tarr	CIC CIC	AIG	110	ACG ACG	110	UAG VAG	AUU	776	TTT	CGG	GA2	GGT	CGG	GGG	TAG	CIC	TTT	فآفالا ا
Gry	LYS	GIU	ıyr	Lys	cys	ъуs	vai	ser	ASN	цуs	ALA	Leu	FIO	ata	PTO	TTe	GTR	Lys	ALL >
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MIC TO	200	MAA	GCC	AAA	GGG	CAG	~~~	CGA	GAA	CCA	CAG	GIG	TAC	ACC	CIG	000	CCA	100	
ברד קוד	Ser	Tare	فافل مالا	TAF	CCC 614	GIC Cl-	فاقاق محط	SCT N	CTT CTT	GGT Dec	GIC Gl-	CAC V-1	AIG	TGG	GAC	GGG	GGT 	AGG	
		uya	nid	Lys	GTA	GTU	ETO	лg	914	ELO	Gru	var	TAL	1.111	Leu	FIO	110	3er	wrg>

Fig.14C.

1090 1100 1110 1120 1130 1140 * * GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT CCG AAG ATA GGG TCG Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser> 110. + * 1170 1190 1200 1150 1160 * * * * * * * * * 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 1230 1210 1220 * * * * * * * * * * * 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> 1290 1320 1270 1300 1310 1280 * * * * * * AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His>

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 ***>

1340

1330

60

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Fig.15A.

20 10 ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GGG CTG CTC AGC TGT CTG CTT CTC TAC CAG TOG ATG ACC CTG TGG COC CAG GAC GAC ACG CGC GAC GAG TOG ACA GAC GAA GAG Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu> 120 70 80 110 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> 170 180 130 140 150 160 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> 240 190 200 210 220 230 - * . ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT GGA AAA CGC ATA ATC TGA CAA TGA AAT TIT TIC AAA GGT GAA CIG TGA AAC TAG GGA CTA CCT TIT GOG TAT TAG Thr Val Thr Leu Lys Lys Fhe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile> 250 260 270 280 290 300 ٠ * . * * 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> 340 350 360 310 330 320 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> 420 370 380 390 400 410 ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA OGC 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 COG Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly> 480 430 440 450 460 470 CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG AGA GTT CAA ATG ACC STA TGA GAA CAS GAS 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> 490 500 510 520 530 540 * 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.

550 560 570 580 590 600 * * * 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> 610 620 630 640 650 660 * * * 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 Asm Thr Ser> 670 680 690 700 710 720 * * * 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> 730 740 750 760 770 780 * * * TGC CCA COG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro> 790 800 810 820 830 B40 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> 850 860 870 880 890 900 × * * GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT CAC TOG 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> 910 920 930 940 950 960 ٠ 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> 970 980 990 1000 1010 1020 * . -CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC GAG TEG CAE GAC GTE GTC CTE ACC GAC TTA CCG TTC CTC ATE TTC ACE TTC CAE AGE TTE Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn> 1030 1040 1050 1060 1070 1080 * * * *

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.

1090 1130 1100 1120 1110 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 COG AAG ATA GGG TOG CTG TAG CGG CAC CTC ACC CTC TOG TTA COC Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly>

1210 1220 1230 1240 1250 1260 * . * ٠ * * CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe>

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

1340 1360 * * 1370 1380 1350 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 TIT ACT Gly Lys ***>

Fig.16A.

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May 20, 2008

Sheet 26 of 55

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Fig.16C.

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Trp	Glu	Ser	: Asr	n Gly	, Glr	n Pro	Glu	i Asr	Asr.	Тут	: Lys	Thr	Thi	Pro	Pro	o Val	Leu	Asp	> Ser>
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CIG	CCG	, AGO	AAC	S AAC	GAG	ATG	; TCC	TTC	GAG	TGC	G CAC	CTG	TT	TCC	TCC		GT	GT	222

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

Fig.16D.

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

 1690
 1700

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 CTC TCC CTG TCT CCG GGT AAA TGA

 GAG AGG GAC AGA GGC CCA TTT ACT

 Leu Ser Leu Ser Pro Gly Lys ***>



.18. Fia 0.4 - Unmodified Flt-1(1-3)-Fc ··· \diamond ··Mut1 : Flt-1(1-3 $_{\Delta B}$)-Fc --O·Mut2 : Flt-1(2-3 $_{\Delta B}$)-Fc -- Δ ·Mut3 : Flt-1(2-3)-Fc 0.3 0.2 0.1 Ω 0 0.1 100 10 1000 nM Fig.19. 0.5 Fit-1(1-3)-Fc COS supe $\begin{array}{c} \hline & \neg & \neg & \neg & Mut1 : Flt-1(1-3_{\Delta B})-Fc \ COS \ supe \\ \hline & \neg & \neg & \neg & Mut2 : Flt-1(2-3_{\Delta B})-Fc \ COS \ supe \\ \hline & 0.4 \ \hline & \neg & \neg & Mut3 : Flt-1(2-3)-Fc \ COS \ supe \\ \end{array}$ 0.3 В 0.2 0.1 0 0.01 0.1 i 10 µg/ml



	Fig	.21A.			>EcoR]	_site		
	10	20	30	40	50	60	70	80
GCTIC	GGCTGCA	GTCGATCGA	CTCTAGAGGA	TCGATCCCCG	GCGAGCTO	AATTOGCAA	CACCATGGTC	AGCTAC
CGAAC	CCCGACGT	CAGCTAGCI	GAGATCTCCL	AGCTAGGGGC	CCGCTCGAG	TTAAGOGTI	GIGGIACCAG	ICGAIG
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	80	100	110	120	130	1401	150	160
2022	NECESSION OF	COLOR COLOR	12012011201120 ADM:0201120120	GCTGTCTGCT	TCTCACAGG	ATCTACTICC	GGAGGTAGACC	TTICGT
CCTG	TGGCCCCA	GGACGACAC	CGCGACGAGI	CGACAGACGA	AGAGTGTCC	TAGATCAAGG	CCTCCATCIGG	AAAGCA
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	250	260	270	280	290	300	310	320
TAACA	TCACTGTI	ACTTTAAAA	AAGTTTCCAC	ITGACACTTI	GATCCCTGAI	GGAAAACGC/	TAATCTGGGA	lagtaga
SATIGI	PAGTGACAA	TGAAATTTT	TTCAAAGGTG	AACTGTGAAA	TAGGGACTA	CCTTTIGCG?	TATTAGACCCT	JTCATCT
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ATTCIC	TTTGATA	agtgtgtgtag	CIGTIGGIT	ATGITAGIAT	CTACACCAA	EACTCAGGCA	<u> Bagtaccttaa</u>	CTIGATA
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Fig.21B. 490

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Fig.21C.

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GACT	FACC	GTT	CCTC	ATGT	TCA	CGT.	TCC	AGAC	GT	GTTTC	:GGG	AGG	GICG	GGG	GTA	GCT	TTT	IGG.	IAGA	GGTI	TCGC	IT
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TCCC	GIC	GGGG	CICI	TGGI	GTC	CAC:	ATG	TGG	GAC	GGGGG	IAGG	GCC	CTAC	TCG	ACT	GGT	TCTI	GGT	CCAG	TCGC	JACTO	GA
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CGGA	CCA	GTTI	CCG	AGA!	IAGO	GTC	GCT	GTA	GCG	GCACC	TCAC	2001	ICICO	STTA		GTC	GGCC	TCT	TGT	IGAT	GTTC	IGG
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CETETEGECCC	AGGACGACACG	CGCGACGACI	ICGACAGACG	AAGAGTGTCC	TAGATCAAGG	CTCCATCIG	GAAAGCA
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NCCCGAAGTAGT	'ATAGTTTACG'	FIGCATGITT	CTITATCCC	SAAGACTGGA	CACTTCGTTGT	CAGTIACCCC	TAAACAT
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FICIGITIGATA K T N Y	420 CTCACACATO GAGTGTGTGTAG L T H I FLTI IG D	430 EACAAACCAA CTGTTIGGTI R Q T N MAIN 2	440 TACAATCAT ATGTTAGTA TIII	450 AGATATCCAG ICTATAGGTC D> >	460 CTGTTGCCCAG GACAACGGGTC	470 GAAGICGCIX CTTCAGCGA(480 XGAGCTGC XCTCGACG
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TTCTGTTTGATA K T N Y	420 CTCACACATCO GAGTGTGTAGO L T H 1 _FLT1 IG DO	430 BACAAACCAA TIGTTIGGTI R Q T N DMAIN 2	440 TACAATCAT ATGTTAGTA T I I 	450 AGATATCCAG ICTATAGGTC D> > I Q	460 CTGTTGCCCAG GACAACGGGTC L L P F	470 GAAGTCGCTK CTTCAGCGAC	480 XGAGCTGC XCTCGACG E L> 137
Fig.22B.

F	ig.2	22B	} .														
490	-	500		510		520		53	0		540			550			560
GGTAGGGGA	GAAGCT	GTCCTC	CAAC	IGCAC	CGTG	rgggc	IGAG	ITTAA	CICA	GGT	GTCA	CCTI	TGA	CTG	GGAC	CTAC	CCA
CCATCCCCT	CTTCGA	CAGGAC	STTG	ACGTG	GCAC	ACCCG	ACTC	AAATT	GAGI	ICCA	CAGT	GGAA	VACI	GAC	CCIC D	SATG	GGT
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97( AGCCGCGGGG4 ICGGCGCCC7	0 AGGAGCA ICCTCGI	980 AGTACAA ACATGTT		990 ACGTI TGCA	ACCG1 IGGC2 V R	100 GTGG CACC	D ICAG( AGTC(	10 CGTCC SCAGG	10 ICAC AGIG	CGT	102 CCTG GGAC	D CACC GTGG	AGG	103 ACTO		IGAA ACTI	1040 TGG0 ACC0
97( AGCCGCGGGA ICGGCGCCCT ( P R I	0 AGGAGCA ICCTCGI E E Ç	980 Agtacaa Acatgtt Q Y N	CAGC GTCG I S	990 ACGT/ TGCA T	ACCG1 IGGC2 Y R	100 IGTGG ACACC V	0 ICAGO AGICO V S	10 CGTCC SCAGG V	10 ICAC AGIG L I	CGT GCA V	102 CCTG GGAC L	D CACC GTGG H	agg TCC Q	103 ACTO TGAO D 1	D GGCI CCG/ W I	IGAA ACTT L N	1040 TGGC ACCC G 32
97( GCCGCGGG2 CGGCGCCC PRI	0 AGGAGCA ICCTCGI E E Q	980 Agtacaa Acatgtt Q Y N	CAGC GTCC I S	990 ACGT/ TGCA T	ACCG1 IGGC2 Y R FCAC:	100 IGTGG ACACC V V	0 ICAGO AGICO V S ALLO	10 GTCC SCAGG V DTYPE	10 TCAC AGTG L 1	CGT KGCAI	102 CCTG GGAC L	0 CACC STGG H	AGG TCC Q	103) ACTO TGAO D I		rgaa 4ctt L N	104 TGG ACC G 3

# Fig.22C.

AGGA( TCCTC	TAC	-		10	60		10	070			108	0		10	90		1	100			111	0		11	20
TCCTC		AAC	TGC	'AAG	GTC	TCC	AAC/	AAA	GCC	CT	CCCA	GCC	CCC	ATC	GAG	AAA	ACC	ATC	TCC	AAA	GCC	AA	GGC	CAG	cc
	DTA	TTC	ACO	STTC	CAG	AGG	TTG.	TT	CGG	GA	GGGT	CGG	GGG	TAG	CTC	TTT	IGG	TAG	AGG	TTT	CGG	TT	200	GTC	GG
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CGAG	AACC		\GG'	rgt z		ССТ	GCC	coc	ATC	co	GGGZ	TGA	GCT	GAC	CAA	GAA	CCA	GGI	CAG	CCI	GA	CT	;cc	IGGI	ICA
3C'TC'	FTGC	<b>T</b> G			GTG	GGA	CGG	GGG	TAC	GG	ccci	ACT	CGA	CTG	GTT	CTI	GGI	CCA	GTC	GGA	CT	GA	GG	1007	GT
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									_FC4	<u>4</u> C1	 >!	A AI [>C	LLOI	TYPE	3										
	12	90		1	300		1	.31(	_FC/	<u>4</u> C1	: >: 13:	A AI T>C   20	LLOI	TYPE	30			134(	 >		13	50		1	360
TGCT	12: GGA	90 CTC	CGA		300 CTCC		1 	.31( CTC	_FC/ ) CTA:	AC1	: >: 13: CAA(	A Al T>C   20 GCTC		TYPE 13 CGTG	30 GAC	`AAC	1 BAGO	134( 2AG(	) STGC	GCAG	13 3CA	50 GGG	gaa	1 CGT	360 271
TGCT	12: GGA	90 CTC GAG	CGA		300 CTCC BAGC		1 CTTC BAAG	.31( CTC	_FC4 ) STAT	AC1 TAG	: >? 13: CAA0	A AI I>C 20 GCTC CGAC	LLOI CACO	I3 GTG CAC	30 GAC	 	I SAGO	L34( CAGC	) STGC		13 GCA	50 GGG	GAA CTT	1 CGTV GCA	360 CTI GAA
TGCT ACGA V L	12: GGA CCT D	90 CTC GAG S	CGA GCT D		300 CTCC BAGC S	CTTC BAAC F	1 CTTC HAAG F	.31( CTC GA( L	_FC4 ) CTA2 SATX Y	AC1 TAG ATC		A Al I>C 20 GCTC CGAC L	LLOI CACO FTGG T	13 GTC CAC V	30 GAC CTG D	CAAC STTC K	I BAGO CTCO S	L34( CAGC STCC R	) STGC CACC W	GCAG CGTC Q	13 GCA CGT Q	50 560 560 560	GAA CTT N	1 CGT GCA V	360 CTI GAA F
TGCT ACGA V L	12: GGA CCT D	90 CTC GAG S	CGA GCT D	1 CGG GCC G	300 CTCC SAGC	TTC BAAC F	1 CTTC BAAG F	.31( CTC GA( L	_FC/ ) SATA Y	AC1 IAG ATC	- : >: 13: CAA0 :GTT 5 K	A AI I>C 20 GCTC CGAC L	LLOT CACC FTGG T	13 GTC CAC V	30 GGAC CTG D	TAAC TTC K	i SAGO STCC S	L34( CAGC STC( R	) STGC CACC W	GCA( CGTX Q	13 SCA CGT	50 GGG CCC G	GAA CTT N	1 CGT GCA V	360 CT1 GAP F
TGCT ACGA V L	12: GGA CCT D	90 CTC GAG S	CGA GCT D	1. CGG GCC G	300 CTCC BAGC S	CTTC BAAC F	1 CTTC BAAG F	.31( CTC GA( L	_FC/ CTA: SAT/ Y _FC/	IAG ATC S	- : >: 13: CAA0 CGTK 5 K	A Al T>C 20 3CTC CGAC L A Al	LLOI T LLOI	13 GTG CAC V TYPI	30 GAC CTG D	CAAC STTC K	i SAGO STCO S	L34( CAG( STC( R	) STGC CACC W	GCAG CGTX Q	13 3CA CGT Q	50 566 500 6	GAA CTT N	1 CGT GCA V	360 271 3AA F 4
TGCT ACGA V L	12 GGA CCTV D	90 CTC GAG S	CGA GCT D	1 CGG GCC G	300 CTCC BAGC S	CTTC BAAC F	1 CTTC SAAG F	.31( CTC CGAC L	_FC/ ) TTA: SATI Y _FC/	AC1 TAG ATC S	- ; 13: CAA0 CTN K	A AL F>C 20 3CTC CGAC L A AL	LLOI CACC STGC T LLOI	13 GGIG CAC V TYPI	30 GGAC CCTG D 3	CAAC STTC K	I BAGC TTCC S	L34( CAGC STCC R	) STGC CACC W	GCAG CGTC Q	13 3CA Q	50 3GG CCC G	GAA CTT N >N	1 CGT GCA V OCLI	360 271 3AF 5 4
TGCT ACGA V L	12: GGA CCTV D	90 CTC GAG S	CGA GCT D		300 CTCC SAGC S	CTTC GAAC F	1 CTTC SAAG F	.31( CTC GA( L	_FCA ) TTA: SATA Y _FCA	AC1	- ; 13: CAAC CGTK 5 K	A AI F>C 20 GGAC CGAC L A AI	LLOI CACC FIGG T	13 GTG CAC V TYPH	330 GAC CCTG D 3	CAAC STTC K	I BAGO STCC S	L34( CAGK FTCC R	) FIGC CACC W	GCAC CGTV Q		50 366 CCC 6	GAA CTT N >N	1 CGT GCA V OtI	360 CTTI GAA F 4 SJ
TGCT ACGA V L	12: GGA CCTV D	90 CTC GAG S 	CGA GCT D		300 CTCC S S 380	TTC BAAC F	1 CTTC F I CTC1	.31( CCT( CGA( L 139(	_FC/ ) CTA: SATJ Y _FC/ ) ACAJ	AC1 IAG ATC S AC1		A AI F>C 20 3CTC CGA0 L A AI 00 ACA0	LLOT CACC FIGG T LLOT	13 CGTC CCAC V TYPH 14 AGAF	330 GGAC D 3	CAAC STTC K	i BAGO STCC S	134( 2AGC FTC( R 	) STGC CACC W	GCAG CGTV Q	13 3CA CGT Q 14	50 3GG CCC G	GAA CTT N >N	1 CGTV GCAV V OctI 1 SGAG	360 CTI SAF Si 440
TGCT ACGA V L TCAT	12: GGA CCTV D 13 GCTV CGA	90 CTC GAG S 70 CCG GGC	CGA GCT D TGA	1 CGGC GCC G G T G	300 CTCC GAGC S 380 ATGJ TAC:	TTC SAAC F	1 CTTC BAAG F 1 CTC7 GAG4	.31( CTC CGA( CGA( L39) IGCL IGCL	_FC/ ) CTA:: SATJ Y _FC/ ) ACAJ	ΔC1 IAG ATC S ΔC1		A AI F>C 20 SCTC CGAC L A AI 000 ACAC	LLOI CACC FIGC T LLOI CGCJ	13 GTG CAC V TYPI 14 AGAJ	330 GGAC D 3 110 AGAG	CAAC STTC K	i BAGO STCO S	L34( 2AGK STC( R L42) CCC' 3GG	) STGC LACC W	GCAG CGTC Q	13 3CA Q 14 CGG 3CC	50 GGG CCC G 30 GTA CAI	GAA CTT N >N AAT	1 CGT GCA V OctI 1 GAG CTC	360 CTI GAF F 4 440 CGC
TIGCT ACGA V L TICAT	12 GGA CCTV D 13 GCTV CGA	90 CTC EAG S 70 CCG GGC S	CGA GCT D TGA ACT V	1 CGG GCC G G G G G G G T G C S M	300 CTCC SACK S 380 ATG TAC: H 1	TTC BAAC F	1 CTTC F F 1 CTC7 SAG# A I	.31( CT( GA( L 139( IGC)	_FC/ ) CTA: SATJ Y _FC/ ) ACAJ IGT H 1	AC1 IAG ATC S AC1 ACCI IGC N	- J S S S S S S S S S S S S S S S S S S S	A AI I>C 20 SCTC CGAC L A AI 000 ACAC IGTC Y	LLOT CACC FIGG T LLOT CGCF FIGG FIGG	13 GTG CAC V TYPH 14 AGAF CTT ) H	330 GGAC CCTC D 3 110 MGAC CCTC C S	CAAC TTC K SCC: CGG		1340 CAGO FTCO R 1420 CCC ² 3GG	D STGC CACC W U IGTC ACAL	GCA( CGTX Q	13 GCA Q 14 CGG GCC P	50 GGG CCC G 30 GTA G	GAA CTT N >N AAT TTA K 455	1 CGTV GCAV V otI 1 GAG CTC *>	40 360 CTT 3AA F 4 

CCGC GGCG



			Fi	g.,	24	A.													
		1	LD *			20 *			30 *			4	10 *			50 *			6
ATG TAC M	GIC CAG V	AGC TCG S	TAC ATG Y	TGG ACC W	GAC CIG D	ACC TGG T	2000 2000 G	GTC CAG V	CIG GAC L	CTG GAC L	TGC ACG C	GOG OGC A	CTG GAC L	CTC GAG L	AGC TCG S	TGT ACA C	CTG GAC L	CTT GAA L	CT GA L
_*							JUP 17.	ri s.	LGIVAI	- Seq	10EBN	iHi		12_				-	2
		•	70 *			80			90			10	00			110			12
ACA	GGA	TCT	AGT	тœ	GGA	AGT	GAT	ACC	GGT	AGA	œr	TTC	GTA	GAG	ATG	TAC	AGT	GAA	ΓA
TGT T	CCT G STUT	AGA S	TCA S	AGG S	CCT G>	TCA	CTA	TGG	CCA	TCT	GGA	aag	CAT	CIC	TAC	ATG	TCA	CTT	TA
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						27_			_30	_hFi		IG D	ILAMC	N.2_			<u></u>	<u> </u>	_4
		1	30			140			150			1	60			170			18
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GGG	CIT	TAA	TATA	CAC	TAC	ACT	CTT	GGA	AGG	GAG CTTC	CIC	CIC	ATT.		ACC		GIT	ACG	10
P	E	I	I	н	M	T	E	G	R	E	L	v	I	P	C	R	V	T	
41				_45				hFLT	1 IG	DOM	AIN	2		55	<u> </u>			<u> </u>	
		1	90 *		:	200 *			210 *			2	20 *		:	230 *			2
ccr	AAC	ATC	ACT	GTT	ACT	TIA	AAA	AAG	TTT	CCA	CTT	GAC	ACT	TIG	ATC	œ	GAT	GGA	A
GGA	TIG	TAG	TGA	CAA	TGA	TAA	TIT	TIC	AAA	GGT	GAA	CIG	TGA	AAC	TAG	GGA	CTA	CCT	Т
61	N	1	т 	65_	т	L.	h	K FLT1	F IG I	P Doma	L DN 2	D	т	L 75	I	P	D	G	
		2	50		:	260			270			2	80			290			3
CGC	ата	ATC	TGG	GAC	AGT	* ACA	DAA	ar.	* יוויוי	יחיג	ልጥል	גיזדי	። እስጥ	602	2012	- - 772 C	ב ב ב	C 222	ک ا
GCG	TAT	TAG	ACC	CIG	TCA	TCT	TIC	ccc	AAG	TAG	TAT	AGT	TTA	CGT	TGC	ATG	TTT	CIT	E
R 81	I	I	W	D 85	S	R	K	G FT/T1	F TG	I DOMA	I TN 2	S	N	A 95	T	Y	K	E	1
		3	10			320		•	330				40			350			 3
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GGG	CIT GAA	CIG	ACC	TGT ACA	GAA	GCA	ACA	GIC	AAT ATTA	GGG	CAT	TIG	TAT ATTA	AAG	ACA		TAT	CIC	A m
G	L	L	-333 T	C	E	A	T	V	N	G	H	L.	Y	K	T	N	Y Y	L.	1
101_				_105			h	FL/T1	IG	DOMA	IN 2			_115					_1
		3'	70 *		:	380 *			390 *			4	00 *			410 *			4
CAT	CGA	CAA	ACC	AAT	ACA	ATC	ATA	gat	GIG	GTT	CIG	AGT	ccc	TCT	CAT	GGA	ATT	GAA	С
GTA	OCT	GTT	TGG	TTA	TGT	TAG	TAT	CTA	CAC	CAA	GAC	TCA	GGC	AGA	GTA		TAA	CTI	G
н 121	R	Q hF	T LTI	N IG D	T MAND	1 N 2	I	D> 129	>										
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									130			_hFL	K1 I	G DO	MAIN	3			_1

		F	-ic	J.2	24	З.													
		43	0		4	40			450			46	50		4	70			480
TCT	GIT	GGA	GAA .	aag	CTT	GIC	TTA	TAA	IGT	ACA	GCA	aga	ACT	gaa	CTA	AAT	GIG	GGG	ATT
AGA S	CAA V	CCT · G	CTT ' E	TIC K	GAA ' L	CAG V	AAT L	TIA . N	ACA C	TGT T	OGT A	TCT R	TGA T	CTT E	GAT	TTA N	CAC	G	TAA I>
141_	•			145_			r	FIKI	IG	DOMA	<u>л</u> и 3		-	155_					160>
		49	0 *		5	00 *			510 *			52	20 *		5	530 *			540 *
GAC	TTC	AAC	TGG	GAA	TAC	CCT	TCT	TCG	AAG		CAG	CAT	AAG	<b>AAA</b>	CIT	GIA	AAC	CGA	GAC
D	AAG F	TIG N	ACC W	E E	AIG Y	P	AL:A S	AGC	K	H	Q	GIA H	K	K	GAA	V	N	R	
161_			<u> </u>	165_			_hFI	жі і	GD	MAIN	13_			_175					_180>
		55	50 *		5	60 *			570 *			5	B0 *		!	590 *			600 *
CTA	<b>AAA</b>	ACC	CAG	TCT	GGG	AGT	GAG	ATG	AAG	AAA	TTT	TIG	AGC	ACC	TTA	ACT	ATA	GAT	GGT
GAT L	K	TGG	Q	AGA S	G	S	E	M	K	K	F	L	S	TGG	L	TGA	I	D	G
181_				_185_				bFLK	IG	DOM	<b>/IN</b> :	3		_195					_200>
		61	LO *		(	520 *			630 *			6	40 *			650 *			660 *
GTA	ACC	CGG	AGT	GAC	CAA	GGA	TIG	TAC	ACC	TGT	GCA	GCA	TCC	AGI	GGG	CIG	ATG		AAG
V	TGG T	R	S	D	Q	G	L	Y	TGG	C	A	À	S	S	G	L	M	T	K>
201				_205			h	FLKI	IG 1	DOMA	IN 3	<u> </u>		_215	i				_220>
		6	70 *		I	680 *			690 *			7	00' *			710 *			720 *
AAG	AAC	AGC	ACA	TTT	GIC	AGG	GIC	CAT	GAA	AAG	GAC	AAA							TGC
K	N	S	T	F	V	R	V	H	E	K>	CIG		. 197	i Gie	, 191				. 1.20
221			_hFL	K1 I	GDO	MAIN	3			_231	< ת <	ĸ	ጥ	ਸ	ጥ	С	Р	P	0
											232			_hFC	2021	a _			_240>
		7	30 *			740 *			750 *			7	760 *			770 *			780 *
CCA	GCA	CCT	GAA	CIC	CIG	GGG	GGZ	. cog	TCA	GIC	TTC	cro	TI	2 000	c ccz	A A A	A CCC		GAC
GGT	A COL	'GGA P	. CTT E	GAG	GAC		: ככים ק	GGC	AGI S	CAG V	AAG F	GAC	AAC F	GGC P	GG7. P	r TT. K	r GGC P	s TR K	
241				_245				h	FCAC	1 A.				255	;				_260>
		7	90 *			800 *			.810			٤	320 *			830 *			840 *
ACC	CIC	ATG	ATC	TCC	œ	ACC	. cc.	GAG	GIC	ACA	TGC	GI	GR	G GR	GAC	GI	g ago		GAA
TGG T	GAG	; TAC M	TAG T	AGG	GCC	TGG T	GG2 P	A CTC E	CAG V	TGI T		CAC V	CAC V	CAC V	CR D	GCA0 V	5 10 5	g GN H	E>
261			-	_265	-`			Ŀ	FCAC	1 A				_27	š				280>
		8	50 *			860 *			870	)		ł	880 *			890 *			900 *
GAC	<u>~</u>	GAG	GIC	AAG	TIC		TGX	G TAC		GAC			G GA	G GIN	G CA	Г АА ⁴	T GO		GACA
CTG D	GGA P	A CIC E	CAG	FTIC K	: AAG F	, TTC N	ACC W	. AIG Y	V V	. C10 D	G	v V	E CR	v V	C GE H	n TE N	تىن م A	s TT K	T>
281		_	-	_285				}	FCAC	1 A				29!	5				300>

# Fig.24C.

		91	.0 *		9	20 *			930			94	0 *		9	50 *			960 *
AAG	œ	œG	GAG	GAG	CAG	TAC	AAC	AGC	ACG	TAC	œt	GIG	GIC	AGC	GIC	CIC	ACC	GIC	CTG
TTC	GGC	GCC	CIC	CIC	GIC	ATG	TTG	TCG	TGC	ATG	GCA	CAC	CAG	τœ	CAG	GAG	TGG	CAG	GAC
K	P	R	Е	E	Q	Y	N	S	T	Y	R	v	v	S	v	L	T	V	2205
301				_305_				DF	CACI	Α_	<u> </u>	<u> </u>		315_			_		320>
		97	0		9	80			990			100	0		10	10		1	.020
			*			*			*				*			*		_	*
CAC	CAG	GAC	TGG	CIG	AAT	GGC	AAG	GAG	TAC	AAG	IGC	AAG	GIC		AAC	AAA	GCC	CIC	CCA
H	Q	D	W	L	N	G	K	E	Y	ĸ	ĉ	K	v	S	N	ĸ	A	L	P>
321				_325_				}	hFCΔC	1 A				_335_			·		_340>
													~~						
		10.	*		1(	)40 *		-	1050			10	50 *		10	570 *			* 1080
GCC	œc	ATC	GAG	ААА	ACC	ATC	TCC	ааа	GCC	ААА	GGG	CAG	œ	CGA	GAA	CCA	CAG	GIG	TAC
CGG	GGG	TAG	CIC	TTT	TGG	TAG	AGG	TTT	œG	TTT	$\infty$	GIC	GGG	GCT	CIT	GGT	GIC	CAC	ATG
A	P	I	E	K	т	I	S	ĸ	A	K	G	Q	P	R	E	P	Q	v	Y>
341				_345				h	FCAC1	A _				_355_					_360>
		10	90		1	100			1110			11	20		1	130			1140
			*			*			*				*			*			*
ACC	CIG	ccc	CCA	TCC	œG	GAT	GAG	CIG	ACC	AAG	AAC	CAG	GIC	AGC	CIG	ACC	TGC	CIG	GIC
IGG	GAC	GGG	GGT	AGG	GCC		CIC	GAC	TGG	TIC	TIG	GIC	CAG	TCG	GAC	TGG T	ACG	GAC T.	CAG V>
361	<u>.</u>	F	r	365	R	5	-	h	FCAC	LA.	-	Ŷ	v	375	5	*			_380>
										-									
		11	50		1	160			1170			11	80		1	190			1200
ورو		11	50 * ידאיז		1	160 *	ATC	600	1170 *	GAG	TGG	11 GAG	80 *	TAA	1 'GGG	190 *		GAG	1200 *
AAA TTT	. ccc	11 TTC	50 * TAT ATA	· ccc	1 AGC TCG	160 * GAC	ATC	600 000	1170 * GTG CAC	GAG CIC	TGG	11 GAG CTC	80 * AGC	AAT TTA	1 666 . 000	190 * CAG	; ccg	GAG	1200 * AAC TTG
AAA TTT K	. 660 7 006 6	11 TTC AAG F	50 * TAT ATA Y	CCC GGG P	1 AGC TCG S	160 * GAC CIG D	ATC TAG I	GCC COG A	1170 * GIG CAC V	GAG CIC E	TGG ACC W	11 GAG CTC E	80 * AGC TOG S	AAT TTA N	1 666 000 6	190 * CAG GTC Q	CCG GGC P	GAG CIC E	1200 * ; AAC ; TTG N>
AAA TTT K 381	. ccc ? ccc G	11 TTC AAG F	50 * TAT ATA Y	• ccc . GGG . P _385	1 AGC TCG S	160 * GAC CIG D	ATC TAG I	GCC CGG A h	1170 * CAC V FCAC	GAG CIC E 1 A	TGG ACC W	11 GAG CTC E	80 * AGC TCG S	AAT TTA N _395		190 * CAG CAG CTC Q	CCG GGC P	GAG CIC E	1200 * ; AAC ; TTG N> _400>
AAA TTT K 381	4 660 7 006 6	11 TTC AAG F	50 * TAT ATA Y	CCC GGG P _385	1 AGC TCG S	160 * GAC CTG D	ATC TAG I	600 036 A h	1170 * CAC V FCAC	GAG CIC E 1 A	TGG ACC W	11 GAG CTC E	80 * AGC TOS S	AAT TTA N _395	1 666 6 6	190 * CAG CAG CTC Q	CCG GGC P	GAG CTC E	1200 * AAC TTG N> _400> 1260
AAA TTT K 381	4 660 7 006 6	11 TTC AAG F	50 TAT ATA Y	0 CCC . GGG P _385	1 AGC TCG S	160 * GAC CIG D 220 *	ATC TAG I	GCC CGG A h	1170 * CAC V FCAC 1230	GAG CIC E 1 A	TGG ACC W	11 GAG CTC E	80 * TCG 5	AAT TTA N _395	1 666 6 6	190 * CAG CTC Q 250	CCG CGC P	GAG CTC E	1200 * AAC TTG N> _400> 1260
AAA TTT K 381 AAC	G G G G C DAC	11 TTC AAG F 12	50 * ATA Y 10 *	COC GGG P _385	1 AGC TCG S 1 CCT	160 * GAC CTG D 220	ATC TAG I	CIG	1170 * CAC V FCAC 1230 * GAC	GAG CIC E 1 A	TGG ACC W	11 GAG CTC E 12	80 * TOG 5	AAT TTA N _395	1 GGG CCC G 1 : TTC	190 * GTC Q 250	CCG GGC P	GAG CTC E	1200 * AAC TTG N> 400> 1260 *
AAA TTT K 381 AAC TTC	GGC G G C TAC G ATG	11 TTC AAG F 12 AAG TTC	50 TAT ATA Y 10	CCC GGG P 385 ACG TGC	1 AGC TCG S 1 CCT GGA	160 * GAC CTG D 220 * CCC CTG	ATC TAG I GTG CAC	CTG	1170 * CAC V FCAC 1230 * GAC	GAG CIC E 1 A TCC AGG	TGG ACC W	11 GAG CTC E 12 GGC CCC	80 * TOS 5 40 * TOC *	AAT TTA N _395 : TTC ; AAG	1 GGG G G 1 : TTC ; AAG	190 * CAG CAG CAG 250 * CTC CAG *	COG GGC P 	GAG	1200 * AAC TTG N> _400> 1260 * C AAG C TTC
AAA TTT K 381 AAC TTC N 401	G G G C TAC TAC TAC	11 TTC AAG F 12 AAG TTC K	50 TAT ATA Y 10 * ACC TGG	CCC GGG P 385 ACG TGC T 405	1 AGC TCG S 1 CCT GGA P	160 * GAC D 220 * CCC *	ATC TAG I GIG CAC V	GCC CGG A h CTG GAC L	1170 CAC V FCAC CAC 1230 CAC CTG D FCAC	GAG CTC E 1 A TCC AGG S 1 A	TGG ACC W SACC U	11 GAG CTC E 12 GGC G	80 * TOS 5 40 * TOC *	AAT TTA N 395 TTC AAG F 415	1 GGG G 1 : TTC ; AAG F	190 * GTC Q 250 * CTC GAG	CCG GCC P TAC TAC S ATG Y	GAG CTC E AGC TCC S	1200 * AAC TTG N> _400> 1260 * AAG TTC K> _420>
AAA TTI K 381 AAC TTC N 401	G G G TAC ATC	11 TTC AAG F 12 AAG TTC K	50 TAT ATA Y 10 * ACC TGC	CCC GGG P _385 ; ACG ; TGC T _405	1 AGC TCG S 1 CCT GGA P	160 * CIG D 220 * CCC *	ATC TAG I GIG CAC	GCC CGG A h CTG GAC L h	1170 CAC V FCAC 1230 CAC CTG D FCAC	GAG CIC E 1 A TCC AGG S 1 A	TGG ACC W GAC CTG D	11 GAG CTC E 12 GGC G	80 * AGC 5 40 * * AGC S	AAT TTA N 395 TTC AAG F 415	1 GGG CCC G 1 TTC AAG F	190 * GTC Q 250 * CTC GAG	CCG GGC P TAC TAC ATG	GAG CTC E AGC TCC S	1200 * AAC TTG N> 400> 1260 * AAG TTC K> 420>
AAA TTT K 381 AAC TTC N 401	G G G TAC ATG Y	11 TTC AAG F 12 AAG TTC K	50 TAT ATA Y 10 ACC TG TG	COCC GGG P _385 ; AOG ; TGC T _405	1 AGC TCG S 1 CCT GGA P	160 (AC) CTG D 220 * CCC CTG D 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCC 220 * CCCC 220 * CCCC 220 * CCCC 220 * CCCC 220 * CCCC 220 * CCCC 220 * CCCC 220 * CCCC 220 * CCCC 220 * CCCCC 220 * CCCCC 220 * CCCCCC 220 * CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	ATC TAG I GIG CAC V	CTG	1170 CAC V FCAC 1230 CAC CAC D FCAC 1290	GAG CTC E 1 A TCC AGG S 1 A	TGG ACC W GAC CTG D	11 GAG CTC E 12 GGC G G	80 * AGC 5 TOG 540 * TOG AGG 5 AGG 500	TTA N 395 TTC AAG F 415	1 GGG G 1 TTC AAG F	190 * CAG CAG CAG CAG CAG CAG CAG CAG	COG GGC P TAC TAC TAC	GAG CTC E AG TO S	1200 * AAC TTG N> 1260 * AAG TTC K> _420> 1320
AAA TTT K 381 AAC TTC N 401	G G G TAC TAC	11 F 12 AAG TTC K 12	50 * TAI ATA Y 10 * ACC T 70	CCC GGG P _385 ACG TGC T _405	1 AGC TOG S 1 CCT CCT CCT P 1	160 * GAC CTG D 2200 * CCC GGG P 280	ATC TAG I GTG CAC V	CTG	1170 * GTG CAC V FFCAC: 1230 * GAC 200 1290 1290	GAG CTC E 1 A TCC AGG S 1 A	GAC W	111 GAG CTC E 122 GGC G 13	80 * AGC : TOG S 40 * TOG : TOG	AAT TTA N _395 TTC AAG F _415	1 GGG G 1 : TTC ; AAG F 1 1 	190 * CAG CGC Q 250 * CCC CGC * CCC 310 *	CCG F TAC TAC ATG Y	GAG CTC E TCC S	1200 * AAC TTG N> 400> 1260 * CAAG TTC K> 420> 1320
AAA TTTI K 381 AAC TTC N 401	C CCC G G C TAC S ATC Y	11 TTC AAG F 12 AAG TTC K	50 * TAII ATAI Y 10 * ACCC T 70 *	• CCC GGG P 385 ; TGC T 405 ; AAG	1 AGC TCG S 1 CCTT GGA P 1 CCTT GGA	160 * GAC CTG D 220 * CCC P 280 * *	ATC TAG I GTG CAC V	GCC CGG A h CTG CAG L h	1170 * GTG CAC V FCAC: 1230 * GAC D FCAC: 1290 * CAG CAG CAG CAG CAG CAG CAG CAG	GAG CTC E 1 A TCC AGG S 1 A	TGG ACC W CTG D	111 GAG CTC E 122 GGC G 13 CTC G CAG	80 * * AGC 2 TCG 5 S 40 * * CG 2 TCC 2 AGC 2 TCC 2 AGC 2 TCC 2 TCC 2 AGC 2 TCC 2 TCCC 2 TCCC 2 TCC 2 TCC 2 TCC 2 TCC 2 TCC 2 T	AAT TTA N _395 : TTC ; AAG F _415 : TCA		190 * CAG 2 GAG 2 CTCC 3 GAG 3 10 * * * * * * * * * * * * *	COG P C TAC C TAC C GTG C CAC	GAG CTC E TCC S	1200 * AAC TTG N> 1260 * AAG TTC K> 420> 1320 * 3 CAT
AAA TTTI K 381 AAC TTC N 401 CTC GAG	C TAC G G C TAC C TAC Y C TAC Y T C TAC	11 TTC AAG F 12 AAG S TTC K 12 12 C C C V V	50 * TAII ATAI Y 10 * ACCC TGC TGC T 70 * GAC CTC D	- CCC GGG P _385 ; ACG ; TGC T _405 ; TGC ; AAG ; TTC K	1 AGC TCG S 1 CCTT CCTT CCTT CCTT CCTT CCTT CCTT	160 * GAC CIG D 2200 * 2200 * 2000 *	ATC TAG I GTG CAC V	CTG A CTG CTG CTG CTG C C C C C C C C C C C C	1170 * GTG CAC V FCAC: 1230 * GAC 1230 * CAG CAG CAG CAG CAG CAG CAG CAG	GAG CTC E 1 A TCC AGG S 1 A GGG G	TGG ACC W CTG D CTG D	11 GAG CTC E 12 GGC G G 13 CTC C C C C C C C C C C C C C C C C C	80 * AGC : TCG S 40 * : TCC : TCCC : TCCC : TCCC : TCCC : TCCC : TC	AAT N TTC AAG F TCA SS	1 GGG G 1 TTC F 1 TTC F 1 C	190 * CAG 2 GTC Q 2250 * CTC GAG 310 * TCC S	COG COG P C TAC C TAC C TAC C TAC C CAC V	GAG CTC E ACC S CTC S C CTC S C CTC S C CTC C CTC C CTC E C CTC E C CTC E C CTC E C CTC E C CTC E C CTC E C CTC E C CTC E C CTC E C CTC E C CTC E C CTC E C CTC E C CTC E C C C C	1200 * AAC TTG N> _400> 1260 * AAG TTC K> _420> 1320 * * GTA H>
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AAA TTTI K 381 AAC TTCI N 401 CTC GAG L L 421	COCG G COCG G ATCC T C C C C C C C C C C C C C C C C	111 TTC AAG F 122 AAG F 122 AAG C TTC K 122 CAC V V	50 * TATI ATA Y 10 * ACCC TGC TGC TGC TGC TGC D 30 *	- CCC GGG P - 385 TGC T - 405 : AAG : AAG : TGC T C K 425	1 AGC TOG S 1 CCT GGA P 1 CCT TOG S CCT TOG S 1 1	160 * GAC CTG D 220 * CCG P 220 * CCG P 220 * CCG P 220 * CCG P 220 * CCG P 220 * CCG P 220 * CCG P 220 * CCG P 220 * CCG P 220 * CCG P 220 * CCG P 220 * CCG P 220 * CCG P 220 * CCG P 220 * CCG P 220 * CCG P 220 * CCG P 220 * CCG P CCG P CCG P CCG P CCG P CCG P CCG P CCG P CCG P CCG P CCG P CCG P CCG P CCG CCG	ATC TAG I GTG CAC V ACC W	CTG A CTG CAG CAG CAG CAG CAG CAG CAG	1170 * GTG CAC V FCAC: 1230 * GAC CTG D FCAC 1290 * CAG CAG CAG CAG D FCAC 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 1230 * 12 * 1230 * 1230 * 12 * 12 * 12 * 12 * 12 * 12 * 12 * 12 * 12 * 12 * 12 * 12 * 12 * 12 * 12 * 1 * 1	GAG CTC E 1 A TCC AGG S 1 A GGGC G 1 A	CTG3 ACC W CTG D AACC CTG D	11 GAG CTC E 12 GGC G G CCC G CCC G CCC G CCC G CCC G CCC G CCC G CCC G CCC G CCC CCC C CCC C CCC C CCC C CCC C CCC C	80 * AGC 5 40 * TCC 600 * TTC 7 AGC 7 AGC	AAT TTA N 395 A35 F 415 C C C C C C C C C C C C C C C C C C C	1 GGG G 1 : TTC ; AAG F 1 : TTC C ; AAG	190 * CAG Q 250 * CTCC C C C C C C C C C C C C	COS GGC P C TAC C ATG Y C GTC C CAC V	GAC CTC E AGC TCC S S AGC TCC S	1200 * AAC TTG N> 1260 * AAG TTC K> 420> 1320 * 3 CAT C GTA H> 440>
AAA TTTI K 381 AAC TTC N 401 CTC GAG L 421 GAG	GCCI	111 TTC AAG F 122 AAG S TTC K 122 CAC V V 13	50 * TATI ATA Y 10 * ACC TGC TGC TGC TGC TGC TGC TGC TGC TGC T	- CCC GGG P 385 ; 7GC T 405 ; 7GC ; 7GG ; 7GC ; 7GG ;	1 AGC TOG S 1 CCTT GGA P 1 AGC S TOG S 1 1 CCTT TOG S 1 1 CCTT TOG S 1 1 CCTT CCTT CCTT CCTT CCTT CCTT S S	160 * GAC CTG D 220 * CCG P 280 * CCG P 280 * CCG R 340 *	ATC TAG I CAC V TGG CAC V	CTG A CTG CAG CAG CAG CAG CAG CAG CAG CAG CAG CA	1170 * GTG CAC V FCAC: 1230 * GAC CTG D FCAC 2 GTC Q FCAC 1290 * 3 GTC Q 1350 * 4 GTC * * * * * * * * * * * * *	GAG CTC E I A TCC AGG S I A GGG G I A	CTGS ACC W CTG D AAAC CTG D	111 GAG CTC E 122 GGC G G 13 CCC G G CCC G CCC G CCC G CCC C CCC C C C C C C C C C C C C C C C	80 * AGC 5 40 * TCG 5 40 * TCG 5 40 * TCG 5 40 * TCG 5 600 * TCG 5 600 * TCG 5 5 5 6 6 6 7 5 5 6 7 6 7 7 7 7 7 7 7 7 7 7 7 7 7	AAT TTA N _395 : TTC ; AAG ; ACT S _435 ; TCT		190 * CAG Q Q 250 * CTCC GAG C 310 * TCCC 370 * GGS	COS GGC P C TAC C ATG Y C GTG C CAC V V	GAC CTC E ACC TCC S C TCC S C TCC M	1200 * AAC TTG N> 1260 * AAG TTC K> 420> 1320 * GTA H> 440>
AAA TTT K 381 AAC TTC N 401 CTC GAG CTC GAG	GCCI	111 TTC AAG F 122 AAG S TTC K 122 CAC V 13 CCC ACC	50 TATI ATA Y 10 * ATCA TGG T TGG T 70 * GAC CTC D 30 *	- CCC - GGG - 385 - 385 - 385 - 405 - 405 - 405 - 405 - 425 - 425 - 425 - 425 	1 AGC TOG S 1 CCT GGA GGA S 1 CAC S	160 * GAC CTG D 220 * CCC GGG P 280 * CCC R 340 * TAC GGG R	ATC TAG I GTG CAC V V TGG ACC W		1170 * GTG CAC V FCAC: 1230 * GAC CTG D FCAC 1290 * CAG CTG Q FFCAC 1350 * CAG CTG CTG CTG CAC * CAC * * CAC * * CAC * * * * * * * * * * * * *	GAG CIC E 1 A TCC AGG S 1 A GGG G 1 A CC G 1 A CC G C 1 A CC C C C C C C C C C C C C C C C C C	CTG CTG CTG D CTG CTG CTG CTG CTG CTG CTG CTG CTG CTG	11 GAG CTC E 12 GGC G G TC CAG V V 13 CTC CAG V	80 * AGC 5 440 * TCG 5 440 * TCG 5 440 * TCG 5 440 * TCG 5 5 5 5 5 5 5 5 5 5 5 5 5	AAT TTA N _395 TTC AAG F _415 S _435		190 * CAG Q Q 250 * CTCC GAG C 310 * TCCC 3310 * 3310 * 3370 * 3370 *	COS COS P P C C C C C C C C C C C C C C C C C	GAG CTK E AGC TCC S ATC TCC M	1200 * AAC TTG N> $400>$ $1260$ * AAG TTC AAG TC AAG TA AAG AAG AAG AAG AAG AAG AAG AAG
AAA TTT K 381 AAC TTC N 401 CTC GAG CTC CAC	C CCI	11 TTC AAG F 12 AAG TTC K 12 CTC CTC CAC V 13 CTC CTC CCC L	50 * TATI ATPA Y 10 * ACCC TGG TGG TGG TGG CTG D * CACC CTG D * CACC CTG D * CACC TGG TGG TGG TGG TGG TGG TGG TGG TGG T	- CCC - GGG - 385 - 385 - 385 - 405 - 405 - 405 - 405 - 425 - 425 - 425 - 100 -	1 AGC TOG S 1 CCT CCT CCT CCT CCT S CCT S CCC S CCT S CCC S CCT S CCC S CCT S	160 * GAC CIG D 220 * CCC GGG P 280 * * CCC C GGG P 280 * * CCC C GGG P 280 * * CCC C C C C C C C C C C C C C C C	ATC TAG I GTG CAC V TGC T TGC T		1170 * GTG CAC V FCAC: 1230 * GAC CTG D FCAC 200 * CAG CTG Q Q FCAC 13500 * CAC CAC * CAC * CAC * * CAC * * CAC * * * CAC * * * * * * * * * * * * *	GAG CTC E 1 A TCC AGG S 1 A GGG G 1 A CCC G 1 A CCC G 1 A CCC G 1 A CCC G 3 A CCC C C C C C C C C C C C C C C C C C	TGS ACC W CTG D CTG CTG N C CTG N	11 GAG CTC E 12 GGC G G CTC G G CTC CAG V V 13 CTCC S CAG V V	80 * AGC TCG S 440 * TCG * AGG * TCG * AGG * TCG * AGG * CTCG * AGG * CTCG * CTCG * CTCG * CTCG * CG *	AAT TTA N _395 F TTC AAG S _415 S _415 S _435 S TCT S _435 S _435		190 * CAG CAG CAG CAG CAG CAG CAG CAG	COS GCC P C TAC S C GTC C C GTC C C C C C C C C C C C C C	GAG CIC E AGC TOX S ATC AC TAA	1200 * AAC TTG N> 400> 1260 * AAG TTC K> 420> 1320 CAT CAT H> 440> AAG AAG

Fig.25A.



## Fig.25B.











-						_	_	
	1D2Flk1D3.FcΔC1(a) & VEGFR1R2-FcΔC1(a)	VEGF/VEGFR1R2-FcΔC1(a)	0.98	0.94	0.99		0.97 ± 0.02	
	toichiometry of hVEGF165 to Flt	VEGF/FIt1D2FIk1D3.FcAC1(a)	0.93	0.97	-		$0.96 \pm 0.03$	
	Binding St	hVEGF165 (nM)	-	10	50		Average ± StDev	

Fig.28.

US 7,374,758 B2

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Fig.29.









Fig.35.



Fig.36.
50 GRPFVEMYSEIPEIIHMTEGRELVIPCRVTSP <u>N</u> ITVTLKKFPLDTLIPDG
رمان المحتمد المح محتمد المحتمد محتمد محتحد محتمد محتمد محتمد محتحد محتمد محتمد محتمد محتحد مح
150 VVLSPSHGIELSVGEKLVL <u>NC</u> TARTELNVGIDFNWEYPSSKHQHKKLVNR
200 DLKTQSGSEMKKFLSTLTIDGVTRSDQGLYTCAASSGLMTKKNSTFVRVH
250 EKGPGDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD
VSHEDPEVKFNWYVDGVEVHNAKTKPREEQY <u>N</u> STYRVVSVLTVLHQDWLN
GKEYK <u>C</u> KVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL
400 TCL VKGFYPSDIA VEWESNGQPENNY KTTPPVLDSDGSFFL YSKL TVDKS
RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK







Fig.41.

Sheet 54 of 55



#### MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES AND METHODS OF USING THEREOF

This application is a divisional of U.S. patent application Ser. No. 10/009,852, filed Dec. 6, 2001, now U.S. Pat. No. 7,070,959, which is a national stage application of International Application No. PCT/US00/14142, filed May 23, 2000, which claims priority of U.S. Provisional Application 10 Ser. No. 60/138,133, filed Jun. 8, 1999. The disclosures of these 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 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 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 35 provides the protein with its ligand binding characteristic. Binding of a ligand to the extracellular domain generally results in signal transduction which transmits a biological signal to intracellular targets. Often, this signal transduction acts via a catalytic intracellular domain. The particular array of sequence motifs of this catalytic intracellular domain determines its access to potential kinase substrates (Mohammadi, et al., 1990, Mol. Cell. Biol. 11:5068-5078; Fantl, et al., 1992, Cell 69:413-413). Examples of receptors that transduce signals via catalytic intracellular domains include 45 the receptor tyrosine kinases (RTKs) such as the Trk family of receptors which are generally limited to cells of the nervous system, the cytokine family of receptors including the tripartate CNTF receptor complex (Stahl & Yancopoulos, 1994, J. Neurobio. 25:1454-1466) which is also gener- 50 ally limited to the cells of the nervous system, G-protein coupled receptors such as the  $\beta_2$ -adrenergic receptor found on, for instance, cardiac muscle cells, and the multimeric IgE high affinity receptor FceRI which is localized, for the most part, on mast cells and basophils (Sutton & Gould, 55 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, 60 1990, Cell 61:203-212; Schlessinger & Ullrich, 1992, Neuron 9:383-391) and molecular interactions between dimerizing intracellular domains lead to activation of catalytic function. In some instances, such as platelet-derived growth factor (PDGF), the ligand is a dimer that binds two receptor 65 molecules (Hart, et al., 1988, Science, 240:1529-1531; Heldin, 1989, J. Biol. Chem. 264:8905-8912) while, for

example, in the case of epidermal growth factor (EGF), the ligand is a monomer (Weber, et al., 1984, J. Biol. Chem. 259:14631-14636). In the case of the FccRI receptor, the ligand, IgE, exists bound to FccRI in a monomeric fashion and only becomes activated when antigen binds to the IgE/FccRI 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, 15 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 25 number of types of cells such as mast cells and basophils. Mast cells derive from bone marrow pluripotent hematopoietic stem cell lineage, but complete their maturation in the tissue following migration from the blood stream (See Janeway & Travers, 1996, in Immunobiology, 2d. Edition, M. Robertson & E. Lawrence, eds., pp. 1:3-1:4, Current Biology Ltd., London, UK, Publisher) and are involved in the allergic response.

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

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

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

Persistent angiogenesis may cause or exacerbate certain 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 30 have been used to visualize morphological changes at endothelial cell borders in inflamed venules of, for example, the rat trachea. Lectins, such as conconavalin A and ricin, that bind focally to inflamed venules reveal regions of the subendothelial vessel wall exposed by gaps that correspond 35 to sites of plasma leakage (Thurston, G., et al., Am J Physiol, 1996, 271: H2547-62).

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

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

The angiopoietins and members of the vascular endothelial growth factor (VEGF) family are the only growth factors ⁶⁰ thought to be largely specific for vascular endothelial cells. Targeted gene inactivation studies in mice have shown that VEGF is necessary for the early stages of vascular development and that Ang-1 is required for later stages of vascular remodeling. ⁶⁵

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

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

U.S. Pat. No. 5,712,380, issued Jan. 27, 1998 and assigned to Merck & Co., discloses 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 Flt1.

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. **13**A-**13**D (SEQ ID NO:3);

(b) the nucleotide sequence set forth in FIG. **14**A-**14**C (SEQ ID NO:5);

(c) the nucleotide sequence set forth in FIG. **15**A-**15**C (SEQ ID NO:7);

(d) the nucleotide sequence set forth in FIG. **16**A-**16**D (SEQ ID NO:9);

(e) the nucleotide sequence set forth in FIG. **21**A-**21**C (SEQ 60 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 nucleotide sequence of (a), (b), (c), (d), (e), (f), or (g) and which encodes a fusion polypeptide molecule having the biological activity of the modified Flt1 receptor 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-10D** (SEQ ID NO:1) or FIG. **24A-24**C (SEQ ID NO:15), which has been modified by acetylation or pegylation wherein the acetylation is accomplished with at least about a 100 fold molar excess of acetylation reagent or wherein acetylation is accomplished with a molar excess of 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. 30

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-14C** (SEQ ID NO:6); (c) the amino acid sequence set forth in FIG. **15A-15C** (SEQ ID NO:8); (d) the amino acid sequence set forth in FIG. **16A-16D** (SEQ ID NO:10); (e) the amino acid sequence set forth in FIG. **21A-21C** (SEQ ID NO;12); (f) the amino acid sequence set 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 extracellular matrix components in Matrigel.RTM., whereas acetylated Flt1(1-3)-Fc does not bind.

FIG. **3**. Binding of unmodified Flt1(1-3)-Fc, acetylated Flt1(i-3)-Fc, and pegylated Flt1(1-3)-Fc in a BIACORETMbased 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 8

BIACORETM 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. 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 Flti (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 step-30 acetylated 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 or step-acetylated) in the solution to completely bind the 55 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 60 VEGF. However, at 5.0 µg/ml, which is several times greater than a 1:1 stoichiometric ratio, both the Flt1(1-3)-Fc and the step-acetylated Flt1(1-3)-Fc proteins are able to saturate the VEGF, regardless of the degree of acetylation.

FIG. 9. Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc. Balb/c mice (23-28 g) were injected subcutaneously with 4 mg/kg of unmodified or 10, 20, 40, 60 and 100 fold excess samples of step-

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acetylated Flt1(1-3)-Fc (3 mice for unmodified, 10, 20 and 40 fold excess samples and 2 mice for 60 and 100 fold excess samples). The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days and 3 days after injection. The sera were assayed in an ELISA-based assay designed to detect Flt1 5 (1-3)-Fc. The  $T_{max}$  for all of the Flt1(1-3)-Fc proteins tested was at the 6 hour time point but the  $C_{max}$  was as follows: Unmodified Flt1(1-3)-Fc: 0.06 µg/ml; 10 fold excess sample:—0.7 µg/ml, 20 fold excess sample—2 µg/ml, 40 fold excess sample—4 µg/ml, 60 fold excess sample—2 10 µg/ml, 100 fold excess sample—1 µg/ml.

FIG. **10A-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.

FIG. **12**A and **12**B. Hydrophilicity analysis of the amino ¹⁵ 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-14**C. Nucleic acid (SEQ ID NO:5) and deduced amino acid sequence (SEQ ID NO:6) of Mut2-Flt1( $2-3_{\Delta^{B}}$ )-Fc.

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. 25

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

FIG. 17. Binding of unmodified FIt1(1-3)-Fc, basic region deletion mutant Flt1(1-3)-Fc, and Flt1(1-3)_{*R*->*N*} mutant proteins in a BIACORETM-based assay. At the sub-stoichiometric ratio (0.25 µg/ml Flt1(1-3)-Fc of unmodified, acetylated or genetically modified samples vs. 01. µg/ml VEGF), there is insufficient Flt1(1-3)-Fc protein to block binding of VEGF to the Flt1(1-3)-Fc immobilized on the BIACORE[™] chip. At 0.5 µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, the stoichiometric ratio approximates 1:1 and there is an increased ability to block VEGF binding to the BIACORE™ chip. At 1.0 µg/ml of unmodi-40 fied, acetylated or genetically modified Flt1(1-3)-Fc proteins, which is approximately a 10:1 stoichiometric ratio, the FIt1(1-3)-Fc proteins are able to block binding of VEGF to the BIACORE™ chip, but they are not equivalent. Unmodified, acetylated, and Mut1: Flt1 (1-3 $\Delta$ B)-Fc are essentially equal in their ability to block VEGF binding, whereas Mut4: ⁴⁵ Flt1(1-3R->N)-Fc is somewhat less efficient at blocking binding.

FIG. **18**. Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1 ( $1-3_{\Delta B}$ )-Fc, Mut2: Flt1( $2-3_{\Delta B}$ )-Fc, and Flt1(2-3) mutant proteins to Matrigel® coated plates. Unmodified Flt1(1-3)-Fc protein binds avidly to these wells, the Mut3: Flt1(2-3)-Fc 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: Flt1( $1-3_{R->N}$ )-Fc glycosylation mutant protein shows only marginal benefit on the Matrigel assay.

FIG. **19**. Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1  $(1-3_{\Delta B})$ -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- 65 3)-Fc, Mut1: Flt1( $1-3_{AB}$ )-Fc, Mut2: Flt1( $2-3_{AB}$ )-Fc, and Flt1(2-3) mutant proteins. the Cmax for these reagents was

as follows: Unmodified Flt1(1-3)-Fc—0.15  $\mu$ g/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  $\mu$ g/ml.

FIG. **21**A-**21**C. Nucleotide (SEQ ID NO:11) and deduced amino acid sequence (SEQ ID NO:12) of the modified Flt1 receptor termed Flt1D2.Flk1D3.Fc $\Delta$ 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. **24**A-**24**C. Nucleotide (SEQ ID NO:15) and deduced amino acid sequence (SEQ ID NO:16) of the modified Flt1 receptor termed VEGFR1R2-Fc $\Delta$ C1(a).

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 these is 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. 26A-26B. 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. 26A) or 3 and 4 fold molar excess (FIG. 26B) of either transient Flt1D2Flk1D3.FcΔC1(a), stable Flt1D2Flk1D3.FcΔC1(a), or transient VEGFR1R2-FcΔC1
(a). At all modified Flt1 receptor concentrations tested there is complete binding of VEGF165 ligand during the preincubation, resulting in no detectable stimulation of 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.FcΔC1(a) or VEGFR1R2-FcΔC1(a) at a concentration of 1 nM (estimated to be 1000 times higher than the KD of the Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a)/VEGF165 interaction) were mixed 5 with varied concentrations of VEGF165. After incubation, concentrations of the free Flt1D2Flk1D3.FcΔC1(a) in solution were measured. The data shows that the addition of 1 nM VEGF165 into the Flt1D2Flk1D3.FcΔC1(a) solution completely blocks Flt1D2Flk1D3.FcΔC1(a) solution completely blocks Flt1D2Flk1D3.FcΔC1(a) binding to the 10 VEGF165 surface. This result suggested the binding stoichiometry of one VEGF165 molecule per one Flt1D2Flk1D3.FcΔC1(a) molecule.

FIG. **31**. Size Exclusion Chromatography (SEC) under native conditions. Peak #1 represents the 15 Flt1D2Flk1D3.Fc $\Delta$ C1(a)/VEGF165 complex and peak #2 represents unbound VEGF165. Fractions eluted between 1.1 and 1.2 ml were combined and guanidinium hydrochloride (GuHCl)was added to a final concentration 4.5M to dissociate the complex. 20

FIG. **32**. Size Exclusion Chromatography (SEC) under dissociative conditions. To separate the components of the receptor-ligand complex and to determine their molar ratio, 50 .mu.l of dissociated complex was loaded onto a SUPER-OSETM 12 PC 3.2/30 equilibrated in 6M GuHCl and eluted, 25 Peak #1 represents Flt1D2Flk1D3.Fc $\Delta$ C1(a) and peak #2 represents VEGF165.

FIG. 33, FIG. 34 and FIG. 35. Size Exclusion Chromatography (SEC) with On-Line Light Scattering. Size exclusion chromatography column with a MiniDawn on-line light 30 scattering detector (Wyatt Technology, Santa Barbara, Calif.) and refractive index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine the molecular weight (MW) of the receptor-ligand complex. As shown in FIG. 33, the elution profile shows two peaks. Peak #1 represents the 35 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 40 the Flt1D2Flk1D3.Fc $\Delta$ C1(a)/VEGF165 complex at the peak position is 157 300 (FIG. 33), the MW of VEGF165 at the peak position is 44 390 (FIG. 34) and the MW of R1R2 at the peak is 113 300 (FIG. 35).

FIG. 36. Peptide mapping and glycosylation analysis. The 45 disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) (SEQ ID NO:12) were determined by a peptide mapping method. There are a total of ten cysteines in Flt1D2.Flk1D3.Fc $\Delta$ C1(a); six of them belong to the Fc region. Cys27 is disulfide bonded to Cys76. Cys121 50 is 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, it can not be determined whether disulfide bonding is occurring between same cysteines (Cys211 to 55 Cys211, for example) or between Cys211 and Cys211. Cys216 is disulfide bonded to Cys306. Cys 352 is disulfide bonded to Cvs410.

There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.Fc $\Delta$ C1(a) (SEQ ID NO:12) and are found to 60 be glycosylated to varying degrees. Complete glycosylation is observed at Asn33, Asn193, and Asn282. Partial glycosylation is observed on Asn65 and Asn120. Sites of glycosylation are highlighted by underline in the Figure.

FIG. **37**. Pharmacokinetics of Flt1(1-3)-Fc (A40), 65 Flt1D2.Flk1D3.Fc $\Delta$ C1(a) and VEGFR1R2-Fc $\Delta$ C1(a). Balb/c mice were injected subcutaneously with 4 mg/kg of

CHO transiently Flt1(1-3)-Fc (A40), expressed CHO Flt1D2.Flk1D3.Fc $\Delta$ C1(a), stably expressed Flt1D2.Flk1D3.Fc\DeltaC1(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). The Tmax for Flt1(1-3)-Fc (A40) was at 6 hrs while the Tmax for the transient and stable Flt1D2.Flk1D3.FcΔC1(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\DeltaC1(a) and VEGFR1R2-Fc\DeltaC1 (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 transiently expressed Flt1D2.Flk1D3.FcΔC1(a) and CHO transiently expressed Flt1D2.VEGFR3D3.FcΔC1(a). The mice were tail bled at 1, 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1 (a). Flt1(1-3)-Fc (A40) could no longer be detected in the serum after day 5 whereas Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(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.Fc $\Delta$ C1(a) and Flt1D2.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.

FIG. **42A-42**B. Assessment of Corpus Luteum Angiogenesis Using Progesterone as a Readout. PMSG was injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats, resulting in a fully functioning corpus luteum containing a dense network of blood vessels that secretes progesterone into the blood stream to prepare the uterus for implantation. The induction of angiogenesis in the corpus luteum requires VEGF. Resting levels of progesterone are about 5 ng/ml and can be induced to 25-40 ng/ml after PMSG. Subcutaneous injection of Flt1(1-3)-Fc (A40) or Flt1D2.Flk1D3.Fc $\Delta$ C1(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 pharmacoki-5 netic 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 10 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 15 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 20 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 intracel- 25 lular 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, 30 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 35 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, 40 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- 45 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 50 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 5 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 65 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 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 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 55 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 60 binding to VEGF.

Expression vectors containing the chimeric nucleic acid molecules described herein can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising

30

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

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

The chimeric polypeptide molecules may be purified by any technique which allows for the subsequent formation of a stable, biologically active chimeric polypeptide molecule. For example, and not by way of limitation, the factors may 25 be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively 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 may be used.

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

The present invention also has diagnostic and therapeutic 50 utilities. In particular embodiments of the invention, methods of detecting aberrancies in the function or expression of the chimeric polypeptide molecules described herein may be used in the diagnosis of disorders. In other embodiments, manipulation of the chimeric polypeptide molecules or agonists or antagonists which bind the chimeric polypeptide molecules may be used in the treatment of diseases. In further embodiments, the chimeric polypeptide molecule is utilized as an agent to block the binding of a binding agent to its target. 60

By way of example, but not limitation, the method of the invention may be useful in treating clinical conditions that are characterized by vascular permeability, edema or inflammation such as brain edema associated with injury, stroke or tumor; edema associated with inflammatory disorders such 65 as psoriasis or arthritis, including rheumatoid arthritis; asthma; generalized edema associated with burns; ascites

and pleural effusion associated with tumors, inflammation or trauma; chronic airway inflammation; capillary leak syndrome: sepsis:

kidney disease associated with increased leakage of protein; and eye disorders such as age related macular degeneration and diabetic retinopathy.

An amino acid sequence analysis of Flt1(1-3)-Fc revealed the presence of an unusually high number (46) of the basic amino acid residue lysine. An IEF analysis of Flt1(1-3)-Fc showed that this protein has pl greater than 9.3, confirming the prediction that the protein is very basic. It was hypothesized that the basic nature of Flt1(1-3)-Fc protein was causing it to bind to extracellular matrix components and that this interaction might be the cause of the extremely short detectable circulating serum half-life exhibited by Flt1(1-3)-Fc when injected into mice. In order to test this hypothesis, Flt1(1-3)-Fc protein was acetylated at the lysine residues to 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, Mass.) and then into 8.5 L roller bottles (Corning, Acton, Mass.) using the cell culture media described supra. Cells were removed from the roller bottles by standard trypsinization and put into 3.5 L of suspension medium. The suspension medium is comprised of glutamine-free ISCHO medium (Irvine Scientific, Santa Ana, Calif.) containing 5% fetal bovine serum (FBS from Hyclone Labs, Logan, Utah), 100 µM MSX and GS supplement (JRH Scientific, Kansas City, Mo.) in a 5 L Celligen bioreactor (New Brunswick Scientific, New Brunswick, N.J.) at a density of  $0.3 \times 10^6$  cells/mL. After the cells reached a density of 3.6×10⁶/mL and were adapted to suspension they were transferred to a 60 L bioreactor (ABEC, Allentown, Pa.) at a density of 0.5×10⁶ cells/mL in 20 L of ISCHO medium with 5% fetal bovine serum. After two days an additional 20 L of ISCHO+5% fetal bovine serum was added

to the bioreactor. The cells were allowed to grow for an additional two days reaching a final density of 3.1×10⁶ 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, 5 Inc., Bedford, Mass.).

#### Example 2

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

Flt1(1-3)-Fc protein was initially purified by affinity chromatography. A Protein A column was used to bind, with high specificity, the Fc portion of the molecule. This affinity- 15 purified protein was then concentrated and passed over a SEC column. The protein was then eluted into the formulation buffer. The following describes these procedures in detail

#### Materials and Methods

All chemicals were obtained from J. T. Baker, Phillipsburg, N.J. with the exception of PBS, which was obtained as a 10.times. concentrate from Life Technologies, Gaithersburg, Md. Protein A Fast Flow and SUPERDEX[™] 200 25 preparation grade resins were obtained from Pharmacia, Piscataway, N.J. Equipment and membranes for protein concentration were obtained from Millipore, Bedford, Mass.

Approximately 40 L of 0.45 µm-filtered CHO conditioned media containing Flt1(1-3)-Fc protein was applied to a 290 30 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 35 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 remove aggregates and other contaminants, the concentrated 40 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 as 50 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. 60 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 pl 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, 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 Dickinson Labware, Bedford, Mass.). The plates are incubated with varying concentrations of either Flt1(1-3)-Fc, 10 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 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 pl of Flt1(1-3)-Fc, but rather by physically shielding the positive charges from interacting with the extracellular matrix. Applicants decided to attempt to improve the pharmacokinetic properties of Flt1(1-3)-Fc molecules by attaching strands of 20K PEGs as described infra.

#### 45 Materials and Methods

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

20K PEG strands functionalized with amine-specific ter-55 minal moieties were used in small-scale reaction studies that were set-up to evaluate different reaction conditions in which the PEG:protein stoichiometry was varied. Based on these reactions and the analyses of samples on standard SDS-PAGE, Flt1(1-3)-Fc at a concentration of 1.5 mg/mL was reacted at pH 8.1 with 20K SPA-PEG (PEG succinimidyl propionate) molecules at a PEG-to-Flt1(1-3)-Fc monomer molar ratio of 1:6. The reaction was allowed to proceed at 8° C. overnight. For initial purification, the reaction products were applied to a 10 mm×30 cm Superose 6 column equilibrated with PBS containing 5% Glycerol. The column appeared to separate pegylated Flt1(1-3)-Fc molecules based on the extent of pegylation. Fractions corresponding to what appeared to be primarily mono-pegylated

and di-pegylated dimeric Flt1(1-3)-Fc, as judged by banding patterns on reducing and non-reducing SDS-PAGE gels were pooled. The protein concentration was determined by measuring absorbance at 280 nm. The pegylated Flt1(1-3)-Fc protein was sterile filtered, aliquoted and stored at  $-40^{\circ}$  5 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,  $_{15}$ unmodified Flt1(1-3)-Fc protein was immobilized on the surface of a BIACORETM chip (see BIACORETM Instruction Manual, Pharmacia, Inc., Piscataway, N.J., for standard procedures) and a sample containing 0.2 µg/ml VEGF and either unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc or 20 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 25 molecule and the Flt1(1-3)-Fc protein is a positively charged molecule, so when the two molecules are mixed together, they should interact through their respective charges. This essentially neutralizes Flt1(1-3)-Fc's inherent positive charge making the molecule behave as if it has been chemically or genetically modified so as to reduce its charge and its tendency to bind via charge interactions. As shown in FIG. 3, acetylated (columns 13-16), pegylated (columns 17-20), and heparin-treated Flt1(1-3)-Fc (columns 21-24) are each able to completely compete with the BIACORETM 35 chip-bound Flt1(1-3)-Fc for VEGF binding as compared to control (columns 1-4) and irrelevant protein (columns 5-8). Unmodified Flt1(1-3)-Fc (columns 5-6) appeared to only partially compete with BIACORE™ chip-bound Flt1(1-3)-Fc for VEGF binding. However, washing the bound samples 40 with 0.5M NaCl (columns 7-8) resulted in a binding profile similar to the modified forms of Flt1(1-3)-Fc, indicating that the unmodified protein was exhibiting non-specific binding to the chip that could be eliminated by the salt wash.

#### Example 7

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

Unmodified, acetylated, and pegylated Flt1(1-3)-Fc proteins were tested in a standard ELISA-based assay to evaluate their ability to bind the Flt1 receptor ligand VEGF. As shown in FIG. **4**, both pegylated and acetylated Flt1(1-3)-Fc proteins are capable of binding to VEGF, demonstrating that 55 modifying the protein either by pegylation or acetylation does not destroy its ability to bind its ligand.

#### Example 8

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

In vivo experiments were designed to assess the pharma- 65 cokinetic profiles of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc protein. Balb/c

mice (23-28 g; 3 mice/group) were injected subcutaneously with 4 mg/kg of unmodified, acetylated, or pegylated Flt1 (1-3)-Fc. The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days, and 3 days after injection of protein. The sera were 5 assayed in a standard ELISA-based assay designed to detect Flt1(1-3)-Fc protein. Briefly, the assay involves coating an ELISA plate with VEGF, binding the unmodified, acetylated, or pegylated Flt1(1-3)-Fc-containing sera, and reporting with an anti-Fc antibody linked to alkaline phosphatase.
10 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 µg/ml-0.15 µg/ml; acetylated: 1.5 µg/ml-4.0 µg/ml; and pegylated: approximately 5 µg/ml.

#### Example 9

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

To determine what minimal amount of acetylation is necessary to eliminate binding to extracellular matrix components, an experiment was designed that acetylated the Flt1(1-3)-Fc protein in a step-wise fashion by using increasing amounts of molar excess of acetylation reagent in the acetylation reaction mixture. The range of molar excess was as follows: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 moles of acetylation reagent per 1 mole of Flt1(1-3)-Fc monomer. The reactions were performed as detailed in the instruction manual provided with the sulfo-NHS-Acetate modification 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-6**B, 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 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.

50 (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-acetylated Flt1(1-3)-Fc (10, 20, and 30 fold molar excess samples), or rTie2-Fc (an irrelevant control) protein were added to the wells. The plates were incubated for 1-2 hours 60 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 dis-10 played no detectable binding, despite the fact that by IEF analysis (FIG. 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 Flt1(1-3)-Fc in a Biacore-Based Assay.

(c.) Binding of Step-Acetylated Flt1(1-3)-Fc in a BIA-  $_{20}$  CORETM-Based Assay

Unmodified and step-acetylated Flt1(1-3)-Fc proteins where tested in a BIACORETM-based assay to evaluate their ability to bind to the Flt1 ligand, VEGF. In this assay, unmodified Flt1(1-3)-Fc protein (0.5, 1.0, or 5.0 µg/ml) was 25 immobilized on the surface of a BIACORETM chip (see BIACORETM 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 30 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 µg/ml VEGF), there is not enough Flt1(1-3)-Fc (either unmodified 35 or step-acetylated) in the solution to completely bind the VEGF. At 1.0 µg/ml, which approximates a 1:1 stoichiometrie 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 40 step-acetylated) to completely bind the available VEGF. However, at 5.0 µg/ml, which is several times greater than a 1:1 stoichiometrie 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 45 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 pharmacokinetic 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 60 (1-3)-Fc (described supra). FIG. 9 details the results of this 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:—0.7 µg/ml, 20 fold molar excess sample—2 µg/ml, 40 fold molar excess sample-4 µg/ml, 60 fold molar excess sample—2 µg/ml, 100 fold molar excess sample—1 µg/ml.

This results demonstrates that acetylation or pegylation of Flt1(1-3)-Fc significantly improves its pharmacokinetic profile.

#### Example 11

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

Based on the observation that acetylated Flt1(1-3)-Fc, which has a pl below 6, has much better pharmacokinetics 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-10**D, in which 6 out of 10 amino acid residues are basic. This sequence is located in Flt1 Ig domain 3 of the receptor (see FIG. **11**), which is not itself essential for binding of VEGF ligand, but which confers a higher affinity binding to ligand. An alignment of the sequence of Ig domain 3 with that of Ig domain 2 reveals that in this region, there is very poor alignment between the two Ig domains, and that there are about 10 additional amino acids in Ig domain 3. An analysis of the hydrophilicity profiles MACVECTOR[™] computer software) of these two domains clearly indicates the presence of a hydrophilic

region in the protein (FIG. 12A-12B). These observations raised the possibility that the actual three dimensional conformation of Flt1 Ig domain 3 allowed for some type of protrusion that is not in Flt1 Ig domain 2. To test this hypothesis, the 10 additional amino acids were deleted and the resulting protein was tested to see whether the deletion would affect the pharmacokinetics favorably without seriously compromising the affinity of the 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 al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene PubI. 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: Flt1(1-3 $\Delta$ B)-Fc construct was derived from Flt1(1-3)-Fc by deletion of nucleotides 814-843 (set forth in FIG. 10A-10D), which deletes the highly basic 10-amino acid residue sequence Lys-Asn-Lys- 20 Arg-Ala-Ser-Val-Arg-Arg-Arg from Flt1 Ig domain 3.

The final DNA construct was sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, Calif.). The sequence of Mut1: Flt1( $1-3_{\Delta B}$ )-Fc is set ²⁵ forth in FIG. **13A-13**D.

#### Example 12

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

A second deletion mutant construct, designated Mut2: Flt1(2-3_{AB})-Fc, was derived from the Mut1: Flt1(1-3_{AB})-Fc construct by deletion of Flt1 Ig domain 1 encoded by nucleotides 79-393 (see FIG. 10A-10D); for convenience, nucleotides 73-78 (TCA GGT) were changed to TCC GGA. This introduced a restriction site (BspE1) without altering the associated amino acid sequence, Ser-Gly. This DNA construct, which was constructed using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. 45 Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, Mass.), was also sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, 50 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 techniques and the final construct was sequence-verified as 65 described supra. The sequence of Mut3: Flt1(2-3)-Fc is set forth in FIG. **15A-15**C.

#### Example 14

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

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

#### Example 15

Characterization of Acetylated Flt1(1-3)-Fc Mut1: Flt1(1-3_{$\Delta B$})-Fc, and Mut4: Flt1(1-3_{R > 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 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_{AB})-Fc protein showed the best profile, binding more weakly than any of the other mutant proteins. The Mut4: Flt1(1-3_{*R*->*N*})-Fc glycosylation mutant protein showed only 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*->*N*}) Fc in a BIACORETM-Based Assay.

Unmodified and acetylated Flt1(1-3)-Fc and genetically modified Mut1: Flt1(1-3 $\Delta$ B)-Fc and Mut4: Flt1(1-3_{*R*->*N*})-Fc proteins where tested in a BIACORETM-based assay to 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 BIACORE™ chip (see BIACORE™ 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 approxi-55 mately (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: FIt1 (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*->*N*})-Fc (at approximately (0.25, 0.5, or 1.0  $\mu$ g/ml) were passed over the Flt1(1-3)-Fc-coated chip. As shown in FIG. 17, at the sub-stoichiometric ratio (0.25 µg/ml Flt1(1-3)-Fc of unmodified, acetylated or genetically modified samples vs. 01. µg/ml VEGF), there is insufficient Flt1 (1-3)-Fc protein to block binding of VEGF to the Flt1(1-3)-Fc immobilized on the BIACORE[™] chip. At 0.5 µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, the stoichiometric ratio approximates 1:1 and there

is an increased ability to block VEGF binding to the BIA-CORETM 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-5 CORETM chip, but they are not equivalent. Unmodified, acetylated, and Mut1: Flt1(1-3AB)-Fc are essentially equal in their ability to block VEGF binding, whereas Mut4: Flt1(1-3_{*R*-5*N*})-Fc is somewhat less efficient at blocking binding. These results confirm the hypothesis that it is possible 10 to reduce the non-specific binding of a positively charged 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,  $_{15}$  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- $3_{AP}$ )-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- $3_{AB}$ )-Fc ₄₀ proteins (4 mice each). These mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days, 3 days, and 5 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc protein which involves coating an ELISA plate with VEGF, binding the Flt1(1-3)-Fc and reporting with an  $_{45}$ anti-Fc antibody linked to alkaline phosphatase. As shown in 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 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 60 explains why unmodified Flt1(1-3)-Fc (described supra) has poor pharmacokinetics that make it difficult to use as a therapeutic agent. As described supra, the chemically modified form of 40 fold molar excess acetylated Flt1(1-3)-Fc, hereinafter termed A40 exhibited a greatly improved phar-65 macokinetic (PK) profile over the non-acetylated Flt1(1-3)-Fc. Therefore, attempts were made to engineer DNA mol-

ecules 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\DeltaC1(a) and VEGFR1R2-Fc\DeltaC1 and R1R3 (Flt1D2.VEGFR3D3-Fc $\Delta$ C1(a) (a) and VEGFR1R3-Fc $\Delta$ C1(a) respectively, wherein R1 and Flk1D2=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 (6519bp) and pMT21.Flk-1(1-3).Fc (5230bp) 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. **21**A-**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. **21**A-**21**C) and continuing into VVLS (corresponding to amino acids 127-130 of FIG. **21**A-**21**C) of Flk1.

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

5': Flt1D2-Flk1D3.s
(5'-ACAATCATAGATGTGGTTCTGAGTCCGTCTCATGG-3')
3': Flk1D3/apa/srf.as
(5'GATAATGCCCGGGCCCTTTTCATGGACCCTGACAAATG-3')

The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused directly to the beginning of Flk1 Ig domain 3, as described above. The 3' amplification primer encodes the end of Flk1 Ig domain 3, defined by the amino acids

VRVHEK (corresponding to amino acids 223-228 of FIG. 21A-21C), followed by a bridging sequence that includes a recognition sequence for the restriction enzyme Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 229-231 of FIG. 21A-21C.

After a round of PCR amplification to produce the individual domains, the products were combined in a tube and subjected to a further round of PCR with the primers bsp/flt1D2 and Flk1D3/apa/srf.as (described supra) to pro-10 duce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 614bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/ΔB2.Fc, to create the plasmid pMT21/Flt1D2.Flk1D3.Fc. The nucleotide sequence of the Flt1D2-Flk1D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 702bp fragment was transferred into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)B2-20  $Fc\Delta C1(a)$  to produce the plasmid pFlt1D2.Flk1D3.Fc $\Delta C1$ (a). The complete DNA and deduced amino acid sequences of the Flt1D2.Flk1D3.Fc\DeltaC1(a) chimeric molecule is set forth in FIG. 21A-21C.

(b) Construction of the Expression pFlt1D2VEGFR3D3Fc∆C1(a)

The expression plasmid pMT21.Flt1(1-3).Fc (6519bp) 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'  35 amplification primers were as follows:

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

3': Flt1D2.VEGFR3D3.as

(TTCCTGGGCAACAGCTGGATATCTATGATTGTATTGGT)

The 5' amplification primer encodes a BspE1 restriction site upstream of Ig domain 2 of Flt1, defined by the amino 45 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 50 were rehydrated with warm DME supplemented with (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 55 for RT-PCR were as follows:

5' · R3D3 s (ATCCAGCTGTTGCCCAGGAAGTCGCTGGAGCTGCTGGTA)

3': R3D3.as (ATTTTCATGCACAATGACCTCGGTGCTCTCCCGAAATCG)

Both the 5' and 3' amplification primers match the sequence of VEGFR3. The 296bp amplification product of this RT-PCR reaction was isolated by standard techniques 65 and subjected to a second round of PCR to add suitable sequences to allow for fusion of the Flt1D2 with the Flk1D3

domains and fusion of the Flk1D3 and Fc domains via a GPG bridge (see below). The amplification primers were as follows:

5': Flt1D2.VEGFR3D3.s (TCATAGATATCCAGCTGTTGCCCAGGAAGTCGCTGGAG)

3': VEGFR3D3/srf.as (GATAATGCCCGGGCCATTTTCATGCACAATGACCTCGGT)

The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' end of VEGFR3 Ig domain 3, defined by the amino acids VIVHEN (corresponding to amino acids 221-226 of FIG. 22A-22C), followed by a bridging sequence that includes a recognition sequence for Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 227-229 of FIG. 22A-22C.

After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain 3) of PCR to produce the individual Ig domains, the PCR products were combined in a tube and Plasmid 25 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 625bp 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 693bp fragment was subcloned into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3) $\Delta$ B2-Fc $\Delta$ C1(a) to produce the plasmid designated pFlt1D2.VEGFR3D3.Fc $\Delta$ C1(a). The complete DNA deduced amino acid sequence of the Flt1D2.VEGFR3D3.FcΔC1(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) 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\DeltaC1(a) starting at 10 nM with subsequent 2-fold dilutions in PBS plus 10% BCS. The plates were then washed 3 times with PBS plus 0.1% Triton-X and incubated with alkaline phosphatase-conjugated anti-human Fc antibody (Promega, 1:4000 in PBS plus 10% BCS) for 1 60 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* DH10B cells carrying the pFlt1D2.Flk1D3.Fc $\Delta$ C1(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 ENDOFREETM 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 NotI 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 cells at a density of 4×10⁶ cells/plate. Plating media was Gibco Ham's F-12 supplemented with 10% HYCLONETM ²⁰ Fetal Bovine Serum (FBS), 100 U penicillin/100 U streptomycin and glutamine (2 mM). The following day each plate of cells was transfected with 6 µg of the pFlt1D2.Flk1D3.Fc∆C1(a) plasmid DNA using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following 25 the manufacturer's protocol. Four hours after adding the transfection mix to the cells, 12 ml/plate of Optimem supplemented with 10% FBS was added. Plates were incubated at 37° C. in a 5% CO₂ incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II supplemented with glutamine (2 mM) and 1 mM sodium butyrate) was added. The plates were incubated at 37° C. for 3 days. After 3 days of incubation, the media was aspirated from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet cells. The supernatant was decanted into sterile 1 L bottles and purification of the expressed protein was performed as described infra.

#### Example 20

### $\begin{array}{c} Construction \ pVEGFR1R2\text{-}Fc\Delta C1C(a) \ Expression \\ Vector \end{array}$

The pVEGFR1R2.Fc $\Delta$ C1(a) expression plasmid was constructed by insertion of DNA encoding amino acids SDT ⁴⁵ (corresponding to amino acids 27-29 of FIG. **24**A-**24**C) between Flt1d2-Flk1d3-Fc $\Delta$ C1(a) amino acids 26 and 27 of FIG. **21**A-**21**C (GG) and removal of DNA encoding amino acids GPG corresponding to amino acids 229-231 of Figure. The SDT amino acid sequence is native to the Flt1 receptor ⁵⁰ and was added back in to decrease the likelihood of heterogeneous N-terminal processing. The GPG (bridging sequence) was removed so that the Flt1 and Flk1 Ig domains were fused directly to one another. The complete DNA and deduced amino acid sequences of the pVEGFR1R2.Fc $\Delta$ C1 (a) chimeric molecule is set forth in FIG. **24A-24C**)

#### Example 21

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

(a) Cell Culture Process Used to Produce Flt1D2.Flk1D3.FcΔC1(a)

The process for production of Flt1D2.Flk1D3.Fc $\Delta$ C1(a) 65 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 greater detail below.

#### Cell Expansion

Two confluent T-225 cm² flasks containing the Flt1D2.Flk1D3.Fc $\Delta$ C1(a) expressing cell line were expanded by passaging cells into eight T-225 cm² flasks in medium (GMEM +10% serum, GIBCO) and incubated at 37° C. and 5% 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° 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 5 L bioreactor (New Brunswick Celligen Plus) where the cells are grown in 3.5 L of suspension culture. The suspension culture medium was a glutamine-free low glucose modification of 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 40 L bioreactor containing the same medium and setpoints for controlling the bioreactor. The temperature setpoint was reduced to 34° C. to slow cell growth and increase the relative rate of protein expression.

Cell Culture Process Used to Produce  $Flt1D2.VEGFR3D3.Fc\DeltaC1(a)$ 

The same methodologies as described supra for  45  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)

The product protein was aseptically harvested from the 55 bioreactor while retaining cells using Millipore Prostak tangential-flow filtration modules and a low-shear mechanical pump (Fristam). Fresh medium was added to the bioreactor to replace that removed during the harvest filtration. Approximately 40 L of harvest filtrate was then loaded onto 60 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 contaminating proteins. Flt1D2.Flk1D3.Fc $\Delta$ C1(a) protein 65 was eluted with a pH 3.0 citrate buffer. The eluted protein was neutralized by addition of Tris base and frozen at  $-20^{\circ}$  C.

25

Several frozen lots of Flt1D2.Flk1D3.Fc∆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% glyc- 10 VEGF165 as compared to control media challenge. erol. The same buffer was used to run the column. The fractions corresponding to Flt1D2.Flk1D3.Fc $\Delta$ C1(a) dimen were pooled, sterile filtered through a 0.22 micron filter, aliquoted and frozen.

(b) Harvest and Purification of Flt1D2.VEGFR3D3.Fc $\Delta$ C1  15 (a)

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

#### Example 23

#### Phosphorylation Assay for Transiently Expressed VEGFR2

Primary human umbilical vein endothelial cells (HU-VECs), passage 4-6, were starved for 2 hrs in serum-free DME high glucose media. Samples containing 40 ng/ml (1 nM) human VEGF165, which is a ligand for the VEGF receptors Flt1, Flk1 and Flt4(VEGFR3) were prepared and 30 were preincubated for 1 hr. at room temperature with varying amounts of the modified Flt1 receptors Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40),  $Flt1D2Flk1D3.Fc\DeltaC1(a)$ and Flt1D2VEGFR3D3.Fc $\Delta$ C1(a) in serum-free DME-high glucose media containing 0.1% BSA. Cells were challenged for 35 minutes with the samples prepared above +/- VEGF165, followed by whole cell lysis using complete lysis buffer. Cell lysates were immunoprecipitated with an antibody directed against the C-terminus of VEGFR2 receptor. The immunoprecipitated lysates were loaded onto 4-12% SDS-PAGE 40 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- 45 26B show the results of this experiment. FIG. 25A-25C reveals that detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are phosphorylated to varying levels depending on which modified Flt1 receptor is 50 used during the preincubations with VEGF. As is seen in FIG. 25A, at a 1.5 molar excess of either Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3.FcΔC1(a) there is complete blockage of receptor stimulation by these three modified Flt1 receptors as compared to control media 55 challenge. In contrast, transient Flt1D2VEGFR3D3.Fc∆C1 (a) does not show significant blockage at this molar excess, as compared with VEGF positive control challenge. Similar results are seen in FIG. 25B, where the modified Flt receptors are in a 3-fold molar excess to VEGF165 ligand. In FIG. 60 25C, where the modified Flt1 receptors are in a 6-fold molar VEGF165 excess to ligand, transient Flt1D2VEGFR3D3.Fc $\Delta$ C1(a) can now be shown to be partially blocking VEGF165-induced stimulation of cell-surface receptors.

In FIG. 26A-26B, detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimu32

lation shows that cell-surface receptors are not phosphorylated by challenge samples which have VEGF165 preincubated with 1 and 2 fold molar excess (FIG. 26A) or 3 and 4 fold molar excess (FIG. 26B) of either transient Flt1D2Flk1D3.Fc $\Delta$ C1(a), stable Flt1D2Flk1D3.Fc $\Delta$ C1(a), or transient VEGFR1R2-Fc∆C1(a). At all modified Flt1 receptor concentrations tested there is complete binding of VEGF165 ligand during the preincubation, resulting in no detectable stimulation of cell-surface receptors by unbound

#### Example 24

#### Cell Proliferation Bioassay

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

 $5 \times 10^3$  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∆C1(a) and Flt1D2.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 1.56 nM VEGF165 with a half maximal dose of 0.8 nM. Flt1(1-3)-Fc and Flt1D2.VEGFR3D3.Fc\DeltaC1(a) are less effective in blocking VEGF165 in this assay with a half maximal dose of ~2 nM. VEGF165 alone gives a reading of 1.2 absorbance units and the background is 0.38 absorbance units

#### Example 25

#### Binding Stoichiometry of Modified Flt Receptors to VEGF165

#### (a) BIACORE[™] Analysis

The stoichiometry of Flt1D2Flk1D3.Fc\DeltaC1(a) and VEGFR1R2-Fc∆C1(a) interaction with human VEGF165 was determined by measuring either the level of VEGF saturation binding to the Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-Fc∆C1(a) surfaces or measuring concentration of VEGF165 needed to completely prevent binding of Flt1D2Flk1D3.Fc $\Delta$ C1(a) or VEGFR1R2-Fc $\Delta$ C1(a) to VEGF BIACORE™ chip surface.
Modified Flt receptors Flt1D2Flk1D3.Fc\DeltaC1(a) and VEGFR1R2-Fc $\Delta$ C1(a), were captured with an anti-Fc specific antibody that was first immobilized on a 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 over the Flt1D2Flk1D3.Fc $\Delta$ C1(a) and VEGFR1R2-Fc $\Delta$ C1(a) surfaces at 10 µl/min for one hour. A real-time binding signal was recorded and saturation binding was achieved at the end 10 of each injection. Binding 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 15 dimeric molecule per one Flt1 D2Flk1 D3.Fc\DeltaC1(a) or VEGFR1R2-Fc  $\Delta$ C1(a) molecule (FIG. 28).

In solution, Flt1D2Flk1D3.Fc\DeltaC1(a) or VEGFR1R2-Fc $\Delta$ C1(a) at a concentration of 1 nM (estimated to be 1000 ₂₀ times higher than the KD of the Flt1 D2Flk1 D3.Fc $\Delta$ C1(a) or VEGFR1 R2-Fc∆C1(a)NEGF165 interaction) were mixed with varied concentrations of VEGF165. After one concentrations of incubation. the free hour Flt1D2Flk1D3.Fc\DeltaC1(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 molar concentration. The data showed that the addition of  $1_{30}$ nM VEGF165 into the Flt1D2Flk1D3.Fc∆C1(a) solution completely blocked Flt1D2Flk1D3.Fc∆C1(a) binding to the VEGF165 surface. This result suggested the binding stoichiometry of one VEGF165 molecule per one Flt1D2Flk1D3.FcΔC1(a) molecule (FIG. 29 and FIG. 30). ³⁵ (c) Calculation of Flt1D2Flk1D3.FcΔC1(a):VEGF165 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.Fc $\Delta$ C1(a) and -1,07 for VEGFR1R2-Fc $\Delta$ C1 ₄₀ (a). The magnitude of the slope, very close to negative one, was indicative that one molecule of VEGF165 bound to one molecule of either Flt1D2Flk1D3.Fc\DeltaC1(a) or VEGFR1R2- $Fc\Delta C1(a)$ .

#### (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 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 col-55 umn run in 6M guanidium chloride. In order to determine complex stoichiometry, several injections of Flt1D2Flk1D3.Fc\DeltaC1(a) and VEGF165 were made and peak height or peak integrated intensity was plotted as a function of the concentration of injected protein. The cali-60 bration was done under condition identical to one used in separating components of Flt1D2Flk1D3.Fc\DeltaC1(a)VEGF complex. Quantification of the Flt1D2Flk1D3.Fc\DeltaC1(a)/ VEGF complex composition was based on the calibration curves. The results of this experiment are set forth in FIG. 65 Flt1D2.Flk1D3.Fc∆C1(a) protein in molar ratio of 3:1 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.Fc\DeltaC1(a)/VEGF165 Complex Preparation

VEGF165 (concentration=3.61 mg/ml) was mixed with CHO cell transiently expressed Flt1D2.Flk1D3.Fc $\Delta$ C1(a) (concentration=0.9 mg/ml) in molar ratio of 3:1 (VEGF165: Flt1D2.Flk1D3.Fc $\Delta$ C1(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 µl of the complex was loaded on a Pharmacia SUPEROSE™ 12 PC 3.2/30 which was equilibrated in PBS buffer. The sample was eluted with the same buffer at flow rate 40 µl/min. at room temperature. The results of this SEC are shown in FIG. 31. Peak #1 represents the complex and peak #2 represents unbound VEGF165. Fractions eluted between 1.1 and 1.2 ml were combined and guanidinium hydrochloride (GuHCl)was added to a final concentration 4.5M 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 µl of dissociated complex as described supra was loaded onto a SUPER-OSE™ 12 PC 3.2/30 equilibrated in 6M GuHCl and eluted with the same solution at a flow rate 40 ul/min. at room temperature. The results of this SEC are shown in FIG. 32.

Complex Stoichiometry

The stoichiometry of the receptor-ligand complex was determined from the peak area or the peak height of the VEGF165 components. Concentrations of 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 µl/min. at room temperature. The standard curve was obtained by plotting peak area or peak height vs protein concentration. The molar ratio of VEGF165:Flt1D2Flk1D3.Fc∆C1(a) determined from the peak area of the components was 1.16. The molar ratio of VEGF165:Flt1D2Flk1D3.FcΔC1(a) determined from the peak height of the components was 1.10.

#### Example 27

#### Determination of the Stoichiometry of the Flt1D2Flk1D3.Fc\DeltaC1(a)/VEGF165 Complex by Size Exclusion Chromatography with On-Line Light Scattering

### **Complex** Preparation

45

VEGF165 was mixed with CHO transiently expressed (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 Barbara, Calif.) and refractive index (RI) detectors (Shi-5 madzu, Kyoto, Japan) was used to determine the molecular weight (MW) of the receptor-ligand complex. Samples were injected onto a SUPEROSE™ 12 HR 10/30 column (Pharmacia) equilibrated in PBS buffer and eluted with the same buffer at flow rate 0.5 ml/min. at room temperature. As  $^{10}\,$ 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- 15 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)/VEGF complex is 1:1 as its corresponds to the sum of molecular weights for Flt1D2Flk1D3.Fc $\Delta$ C1(a) and VEGF165. Importantly, this method conclusively proved that the Flt1D2Flk1D3.Fc $\Delta$ C1²⁵ (a)/VEGF165 complex was indeed composed of only one molecule of VEGF165 ligand and only one molecule of the Flt1D2Flk1D3.Fc $\Delta$ C1(a).

#### Example 28

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

The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.Fc $\Delta$ C1(a) were determined by a peptide mapping method. In this method, the protein was first cleaved with trypsin. Tryptic fragments were analyzed and identified by HPLC coupled with mass spectrometry, in addition to an N-terminal sequencing technique.

Reduction of the tryptic digest was employed to help identify disulfide-bond-containing fragments. Treatment of the tryptic digest with PNGase F (Glyko, Novato, Calif.) was employed to help identify fragments with N-linked glycosylation sites. The results are summarized in the  $_{45}$  accompanying FIG. **36**.

There are а total of ten cvsteines in Flt1D2.Flk1D3.Fc $\Delta$ C1(a); six of them belong to the Fc region. Cys27 has been confirmed to be disulfide bonded to Cys76. Cys121 is confirmed to be disulfide bonded to Cys  $_{50}$ 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 these two cysteines can not be separated enzymatically from each other, it can not be determined whether disulfide 55 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 60 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 65 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.FcΔC1(a), CHO stably expressed Flt1D2.Flk1D3.Fc\DeltaC1(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). The ELISA involves coating an ELISA plate with VEGF165, binding the detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. The results of this experiments are shown in FIG. 37. The  $T_{max}$  for Flt1(1-3)-Fc (A40) was at 6 hrs while the  $T_{max}$  for the transient and stable Flt1D2.Flk1D3.Fc $\Delta$ C1 (a) and the transient VEGFR1R2-Fc $\Delta$ C1(a) was 24 hrs. The  $_{max}$  for Flt1(1-3)-Fc (A40) was 8  $\mu$ g/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),
 30 Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1 (a)

Balb/c mice (25-30 g) were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.Fc\DeltaC1(a) and CHO transiently expressed 35 Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a). The mice were tail bled at 1, 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc, Flt1D2.Flk1D3.Fc\DeltaC1(a) and Flt1D2.VEGFR3D3.Fc\DeltaC1 (a). The ELISA involves coating an ELISA plate with 165, binding the Flt1(1-3)-Fc, Flt1D2.Flk1D3.Fc $\Delta$ C1(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 Flt1D2.Flk1D3.Fc $\Delta$ C1(a) whereas. and Flt1D2.VEGFR3D3.Fc $\Delta$ C1(a) were detectable for 15 days or more. The results of this experiment are shown in FIG. 38.

#### Example 30

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

To evaluate the ability of Flt1D2.Flk1D3.Fc $\Delta$ 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 $\Delta$ C1(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.Fc $\Delta$ C1(a) or vehicle either every other day (EOD) or two times per week

 $(2\times/wk)$  for a period of 2 weeks. After 2 weeks, animals were perfused with fixative, tumors were removed and samples were blinded. Tumor volume was determined by measuring the length and width of visible subcutaneous tumors. Both of Flt1(1-3)-Fc (A40) and 5 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 15 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 2 the reproductive tissues provided the first clear evidence that VEGF acts as a mediator of physiological angiogenesis in mature rodents, as well as humans and non-human primates (Phillips et al, 1990; Ravindranath et al, 1992; Shweiki et al, 1993; Kamat et al, 1995). As cyclic angiogenesis and 25 vascular remodeling are prominent features of the normal ovary and uterus, it is not surprising that abnormal blood vessel growth and/or vascular dysfunction have been found to characterize many pathological conditions which affect these organs. Furthermore, these pathogenic vascular abnor- 30 malities are thought to be caused or perpetuated by the dysregulated expression of one or more angiogenic or antiangiogenic factors, most prominently VEGF.

For example, abnormal angiogenesis is characteristic of polycystic ovary disease, endometriosis and endometrial 35 the carcinoma, and in each case VEGF is over expressed in the affected tissue (Kamat et al, 1995; Shifren et al, 1996; Guidi et al, 1996; Donnez et al, 1998). Overexpression of VEGF is also thought to play a pathogenic role in the establishment of systemic vascular hyperpermeability in ovarian hyper- 40 stimulation syndrome (McClure et al, 1994; Levin et al, 1998) and preeclampsia (Baker et al, 1995; Sharkey et al, 1996). In addition, VEGF has been implicated as the permeability factor responsible for the production of ascites associated with ovarian carcinoma and other tumors (Senger 45 et al, 1983; Boocock et al, 1995). Agents which effectively neutralize the biological actions of VEGF can reasonably be anticipated to be of therapeutic benefit in the above and related conditions.

Angiogenesis and vascular remodeling are also hallmarks 50 of blastocyst implantation and placental development (Findlay, 1986). VEGF is strongly expressed both in the maternal decidua and in embryonic trophoblasts, where it is thought to first stimulate expansion and hyperpermeability of the uterine vasculature during the peri-implantation period and subsequently mediate formation of both the maternal and embryonic components of the placental vasculature (Shweiki et al, 1993; Cullinan-Bove and Koos, 1993; Chakraborty et al, 1995; Das et al, 1997). VEGF is also required for luteal angiogenesis and associated progesterone 60 secretion necessary to prepare the uterus for implantation (Ferrara et al, 1998). Thus, agents which inhibit the biological actions of VEGF may prove to be useful as contraceptive agents (by preventing implantation), or as an abortifacients in the early stages of gestation. The latter application might find particular use as a non-surgical intervention for the termination of ectopic pregnancies.

While the expression of VEGF receptors is largely confined to the vascular endothelium in normal reproductive tissues, Flt1 is also expressed by trophoblasts in the placenta in both humans and animals (Clark et al, 1996; He et al, 1999) where it has been proposed to play a role in trophoblast invasion. Interestingly, both Flt1 and KDR (Flk1) are expressed by choriocarcinoma cell line BeWo (Charnock-Jones et al, 1994), and VEGF has been shown to promote DNA synthesis and tyrosine phosphorylation of MAP kinase in these cells. Furthermore, primary and metastatic ovarian carcinomas not only to express high levels of VEGF, but-in addition to the vascular endothelium-the tumor cells themselves express KDR and/or Flt1 (Boocock et al, 1995). These findings suggest that VEGF may not only be critically involved in the generation and maintenance of tumor vasculature, but that at least in some tumors of reproductive origin VEGF may subserve an autocrine role, directly supporting the survival and proliferation of the tumor cells. Thus agents which block the actions of VEGF may have particularly beneficial applications to the treatment of tumors of reproductive origin.

#### Methods and Results

#### (a) Assessment of VEGF-Induced Uterine Hyperpermeability

Pregnant mare's serum gonadotrophin (PMSG) was injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats. This results in a surge of estradiol after 2 days which in turn causes an induction of VEGF in the uterus. It is reported that this induction results in hyperpermeability of the uterus and an increase in uterine wet weight 6 hrs. later and, therefore, could potentially be blocked by modified Flt receptors Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc\DeltaC1(a) and Flt1D2.VEGFR3D3.Fc\DeltaC1 (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.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. 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 10 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  $_{20}$  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-

 $3_{NAS}$ )-Fc, Flt1(1- $3_{R->C}$ )-Fc and Tie2-Fc. Flt1(1- $3_{NAS}$ )-Fc is a modified version of Flt1(1-3)-Fc in which the highly basic amino acid sequence KNKRASVRRR is replaced by NAS-VNGSR, resulting in the incorporation of two new glycosylation sites and a net reduction of five positive charges, both with the purpose of reducing the unfavorable effects of this sequence on PK. Flt1(1-3_{*R*->*C*})-Fc is a modification in which a single arginine (R) residue within the same basic amino acid sequence is changed to a cysteine (C) (KNK RASVRRR->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∆C1(a). Tie2Fc has no affinity for VEGF165.

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49

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61

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Met	Tyr	Ser 35	Glu	Ile	Pro	Glu	Ile 40	Ile	His	Met	Thr	Glu 45	Gly	Arg	Glu	
Leu	Val 50	Ile	Pro	Суз	Arg	Val 55	Thr	Ser	Pro	Asn	Ile 60	Thr	Val	Thr	Leu	
Lys 65	Lys	Phe	Pro	Leu	Asp 70	Thr	Leu	Ile	Pro	Asp 75	Gly	Lys	Arg	Ile	Ile 80	
Trp	Asp	Ser	Arg	Lys 85	Gly	Phe	Ile	Ile	Ser 90	Asn	Ala	Thr	Tyr	Lys 95	Glu	
Ile	Gly	Leu	Leu 100	Thr	CAa	Glu	Ala	Thr 105	Val	Asn	Gly	His	Leu 110	Tyr	LYS	

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Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln 115 120 125 Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly His Thr Leu Val 135 130 Leu As<br/>n Cys Thr Ala Thr Thr Pro Leu As<br/>n Thr Arg Val Gl<br/>n Met Thr  $% \mathcal{A}$ 155 145 150 160 Trp Ser Tyr Pro Asp Glu Lys Asn Lys Arg Ala Ser Val Arg Arg Arg 170 165 175 Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr 180 185 190 Ile Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val 195 200 205 Arg Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr 210 215 220 Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr 225 230 235 240 Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe 245 250 255 Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro 260 265 270 Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val 275 280 285 Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr 290 295 300 Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val 310 320 305 315 Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys 330 325 335 Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser 340 345 350 Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro 355 360 365 Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val 370 375 380 Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu $\operatorname{Trp}$  Glu Ser As<br/>n Gly 400 385 390 395 Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp 405 410 Gly Ser Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp 420 425 430 Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His 435 440 445 Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys 455 450 460 <210> SEQ ID NO 9 <211> LENGTH: 1704 <212> TYPE: DNA <213> ORGANISM: Homo sapiens <220> FEATURE: <221> NAME/KEY: CDS <222> LOCATION: (1) ... (1701) <400> SEQUENCE: 9

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48

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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	24	0
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	28	8
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	33	6
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	38	4
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	43	2
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ccc Pro	ttg Leu	aac Asn	acg Thr 260	aga Arg	gtt Val	caa Gln	atg Met	acc Thr 265	tgg Trp	agt Ser	tac Tyr	cct Pro	gat Asp 270	gaa Glu	aaa Lys	81	6
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Cys	Gly	Arg	Asn	Gly 85	Lys	Gln	Phe	Cys	Ser 90	Thr	Leu	Thr	Leu	Asn 95	Thr
Ala	Gln	Ala	Asn 100	His	Thr	Gly	Phe	Tyr 105	Ser	Сүз	Lys	Tyr	Leu 110	Ala	Val
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Glu	Leu	Leu 355	Gly	Gly	Pro	Ser	Val 360	Phe	Leu	Phe	Pro	Pro 365	Lys	Pro	Lys
Asp	Thr 370	Leu	Met	Ile	Ser	Arg 375	Thr	Pro	Glu	Val	Thr 380	Суа	Val	Val	Val
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Ile	Ala	Val	Glu 500	Trp	Glu	Ser	Asn	Gly 505	Gln	Pro	Glu	Asn	Asn 510	Tyr	Lya	
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 Pro
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Ile	Ser	Lys	Ala	Lys 325	Gly	Gln	Pro	Arg	Glu 330	Pro	Gln	Val	Tyr	Thr 335	Leu
Pro	Pro	Ser	Arg 340	Asp	Glu	Leu	Thr	Lys 345	Asn	Gln	Val	Ser	Leu 350	Thr	Cys
Leu	Val	Lys 355	Gly	Phe	Tyr	Pro	Ser 360	Asp	Ile	Ala	Val	Glu 365	Trp	Glu	Ser
Asn	Gly 370	Gln	Pro	Glu	Asn	Asn 375	Tyr	Lys	Thr	Thr	Pro 380	Pro	Val	Leu	Asp
Ser 385	Asp	Gly	Ser	Phe	Phe 390	Leu	Tyr	Ser	Lys	Leu 395	Thr	Val	Asp	Lys	Ser 400
Arg	Trp	Gln	Gln	Gly 405	Asn	Val	Phe	Ser	Cys 410	Ser	Val	Met	His	Glu 415	Ala
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25

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Val Val Leu Ser 1

We claim:

**1**. A method of inhibiting vascular endothelial growth factor (VEGF) activity in a mammal, comprising:

administering a pharmaceutical composition to the mammal, wherein the pharmaceutical composition comprises

(a) a VEGF antagonist, and

(b) a pharmaceutically acceptable carrier

- wherein the VEGF antagonist comprises a dimeric fusion polypeptide comprising two fusion polypeptides, each fusion polypeptide comprising: 20
- (i) a VEGF receptor component consisting of an immunoglobulin-like (Ig) domain 2 of a first VEGF receptor human Flt1 and Ig domain 3 of a second VEGF receptor human Flk1 or human Flt4; and

(ii) a multimerizing component,

wherein VEGF activity is inhibited.

2. The method of claim 1, wherein the mammal is a human.

- 10 **3**. A method of inhibiting tumor growth in a mammal, comprising:
  - administering a pharmaceutical composition to the mammal, wherein the pharmaceutical composition comprises
  - (a) a VEGF antagonist, and

(b) a pharmaceutically acceptable carrier

- wherein the VEGF antagonist comprises a dimeric fusion polypeptide comprising two fusion polypeptides, each fusion polypeptide comprising:
- (i) a VEGF receptor component consisting of an immunoglobulin-like (Ig) domain 2 of a first VEGF receptor human Flt1 and Ig domain 3 of a second VEGF receptor human Flk1 or human Flt4; and
- (ii) a multimerizing component,

wherein tumor growth is inhibited.

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