

VHH3 Humanization

SUBSTITUTE SHEET (RULE 26)

seq	10	20	30	40	50
AbM	10	20	30	40	50
VHH3	DVQLVESGGGLVQPGGSLRLSCTSS	ANINSIETLG	WYRQAPGKQRELIA	NMRGGGYMK	
	*	**	*	**	***
3-66*01	EVQLVESGGGLVQPGGSLRLSCAAS	GFTVSSNYS	WVRQAPGKLEWVS	VIYSGGSTY	
hVHH3-H1	EVQLVESGGGLVQPGGSLRLSCAAS	ANINSIETLG	WVRQAPGKLEWVS	NMRGGGYMK	
hVHH3-H2	EVQLVESGGGLVQPGGSLRLSCASS	ANINSIETLG	WYRQAPGKREWIA	NMRGGGYMK	
hVHH3-H3	EVQLVESGGGLVQPGGSLRLSCASS	ANINSIETLG	WYRQAPGKREWIA	NMRGGGYMK	
hVHH3-H4	EVQLVESGGGLVQPGGSLRLSCASS	ANINSIETLG	WYRQAPGKQRELIA	NMRGGGYMK	# ^
	*	**	*	**	***
seq	60	70	80	90	100
AbM	60	70	80	90	110
VHH3	YAGSLKGRFTTMSAKNTMYLQMNSLKPEDTAVYICV	KLRDDDDYV	RGQGTQVTVSS	(SEQ ID NO: 382)	
	*	*	*	*	*
3-66*01	YADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYICAR				
hVHH3-H1	YADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYICAR	KLRDDDDYV	RGQGTQVTVSS	(SEQ ID NO: 430)	
hVHH3-H2	YADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYICAR	KLRDDDDYV	RGQGTQVTVSS	(SEQ ID NO: 431)	
hVHH3-H3	YADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYICV	KLRDDDDYV	RGQGTQVTVSS	(SEQ ID NO: 383)	
hVHH3-H4	YADSVKGRFTISRDNKNTLYLQMNSLRAEDTAVYICV	KLRDDDDYV	RGQGTQVTVSS	(SEQ ID NO: 384)	
	*	*	*	*	*

FIG. 2A

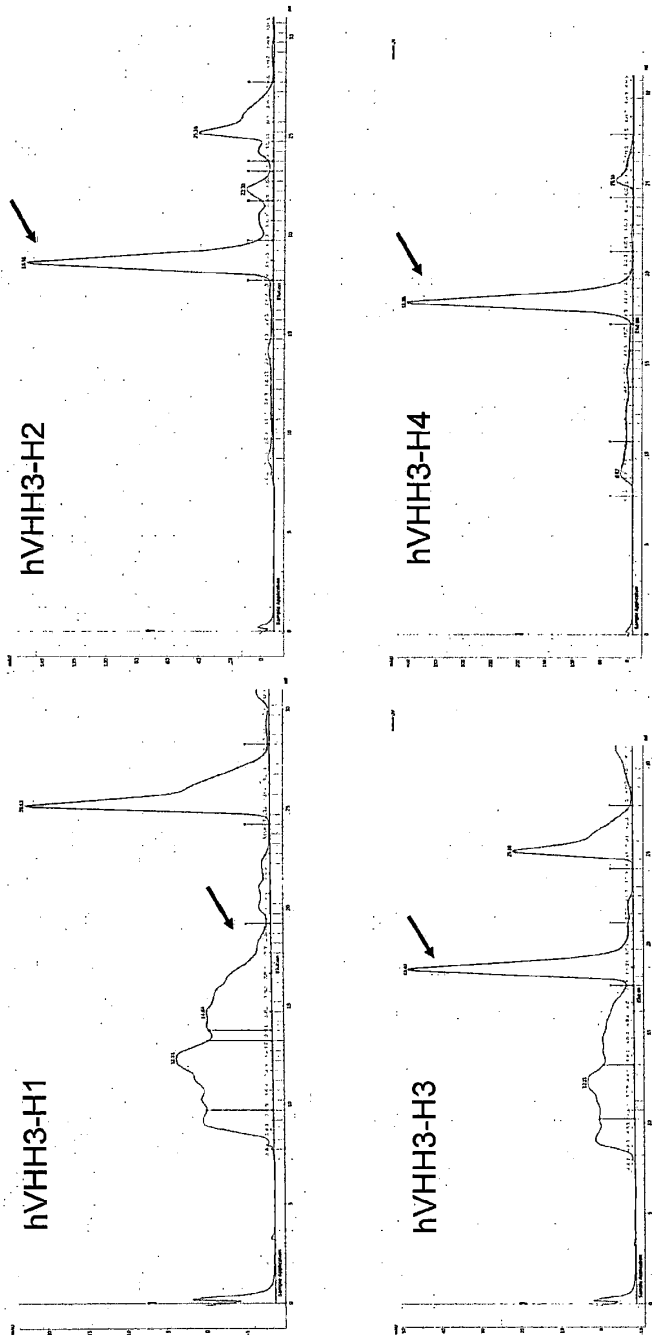


FIG. 2B

De-risk N29 Deamination Motif NS in hVHH3-H4

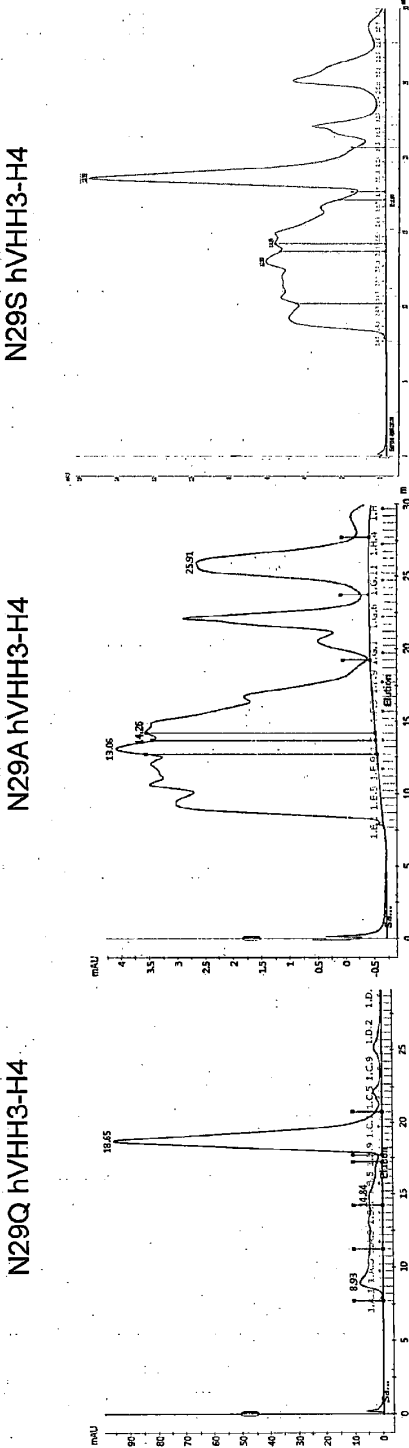


FIG. 3

De-risking of Isomerization Motifs with in DDD sequence of hVHH3-H4

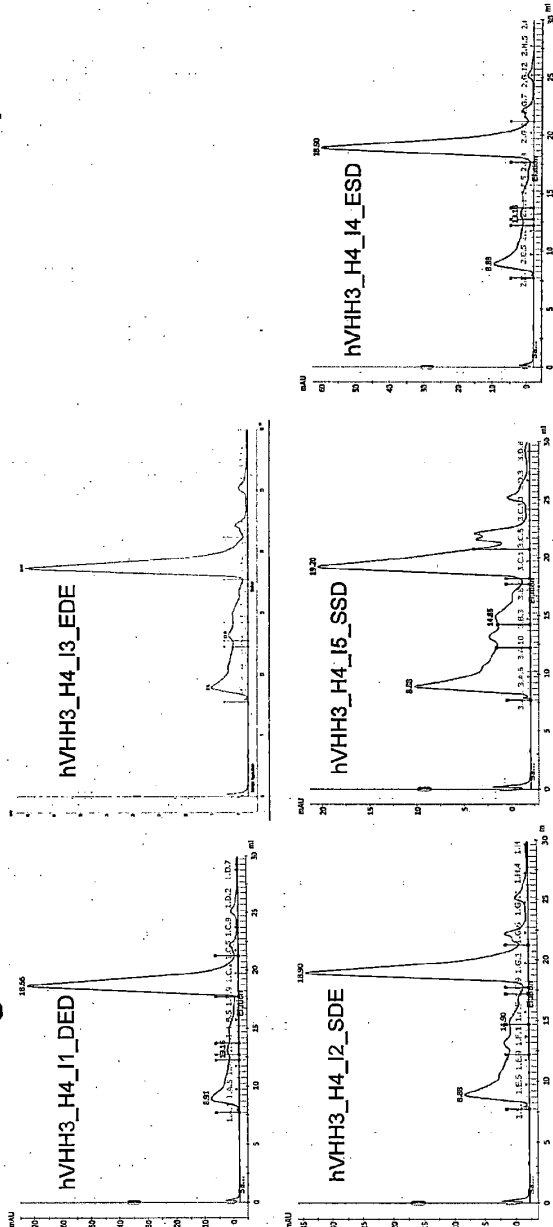


FIG. 4

Oxidation Sensitive Residues of VHH3 cannot be De-risked

Protein	Yield
VHH3	100%
VHH3_M51K	<5%
VHH3_M51L	15%
VHH3_M51I	10%
VHH3_M57K	<5%
VHH3_M57L	10%
VHH3_M57I	10%

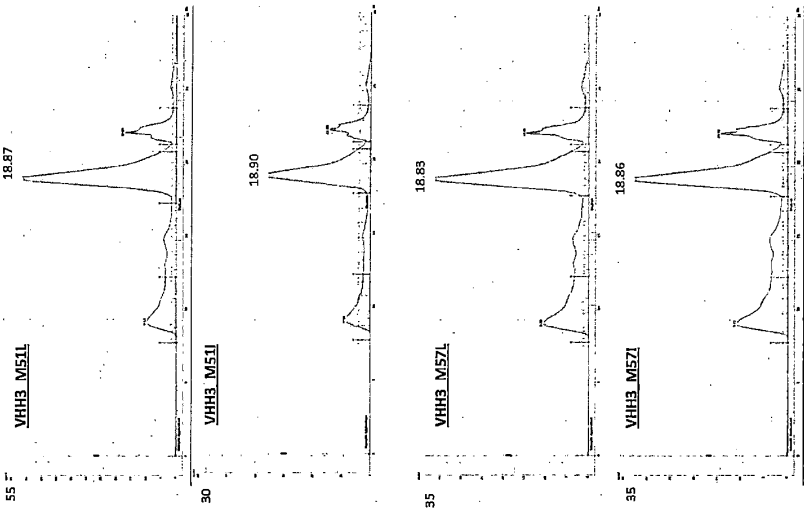


FIG. 5

De-risking of Liable Residues on CDR Loops 4SD1-03_LALAPG

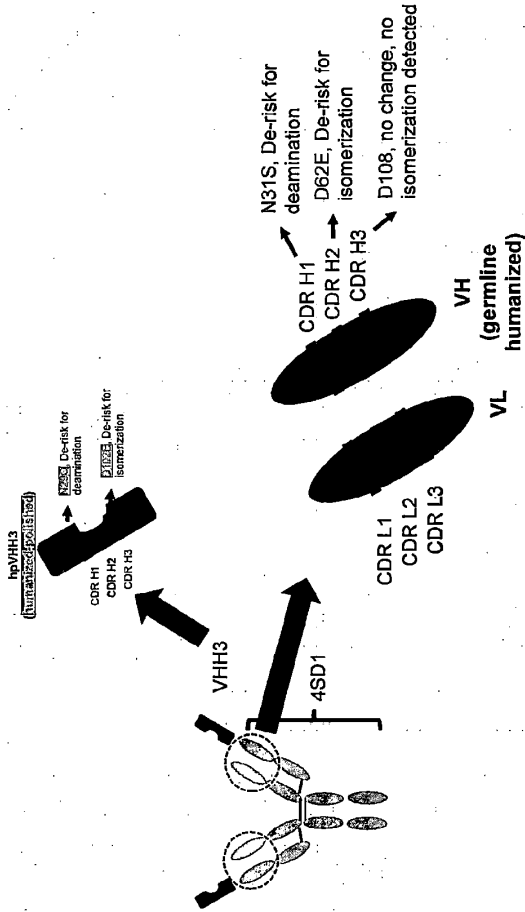


FIG. 6

The Liability Amino Acids in 4SD1 (CDR)

4SD1_LC (the anti-Fzd4 VL portion)

DIQMTQSPSSLSASVGDRVTTCRASQSISSYLNWYQQKPGKAPKLLIYAASLQSGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQS
YSTPLIFGGGTKVEIK

(SEQ ID NO: 281)

Residue 62

4SD1_HC (the anti-Fzd4 VH portion)

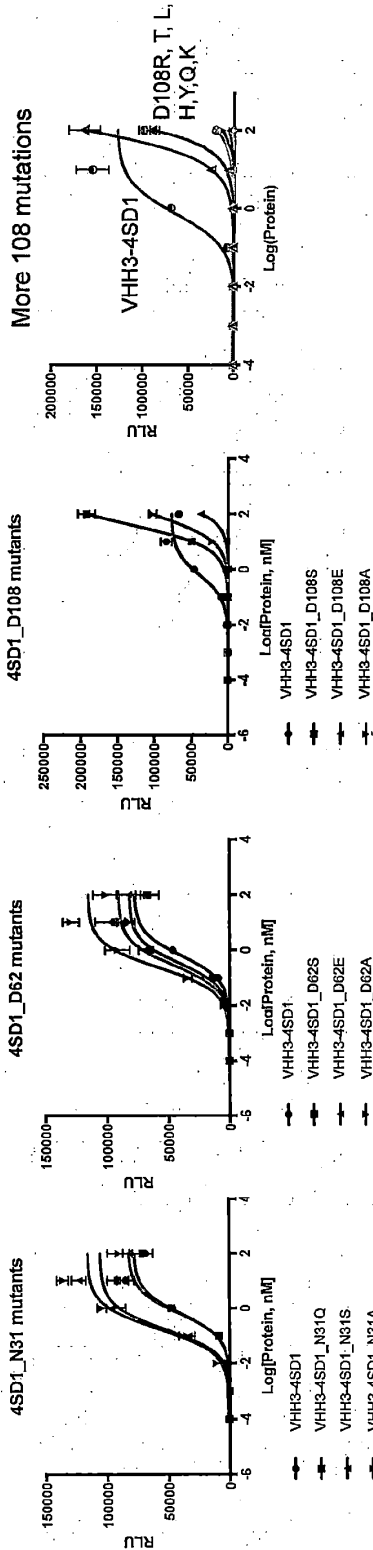
EVQLVESGGGLVKPGGSLRLSCAASGFTFTNYAMSWVRQAPGKGLEWWSAISGSGGTYADSVKGRFTISRDDSKNTLYLQMNSLKTE
DTAVYCARATGFGTIVFDYWGQGLTVYSS

(SEQ ID NO: 182)

Residue 31

Residue 108

CDR_H1: NY: Q, S, A
CDR_H2: DS: E, S, A
CDR_H3: DY: E, S, A



- Mutants of CDR1 N31 and CDR2 D62 on 4SD1 VH have either similar or higher STF Activity comparing to the parental molecule.
- D108 on CDR3 of 4SD1 VH cannot be replaced.

FIG. 7

Framework Mutations on 4SD1

										Frame work		4SD1 VH	IGHV3-23*04
										FR1	K		Q
										FR2	-		
										FR3	D, KT		N, RA

IGHV3-23*04
Homo sapiens

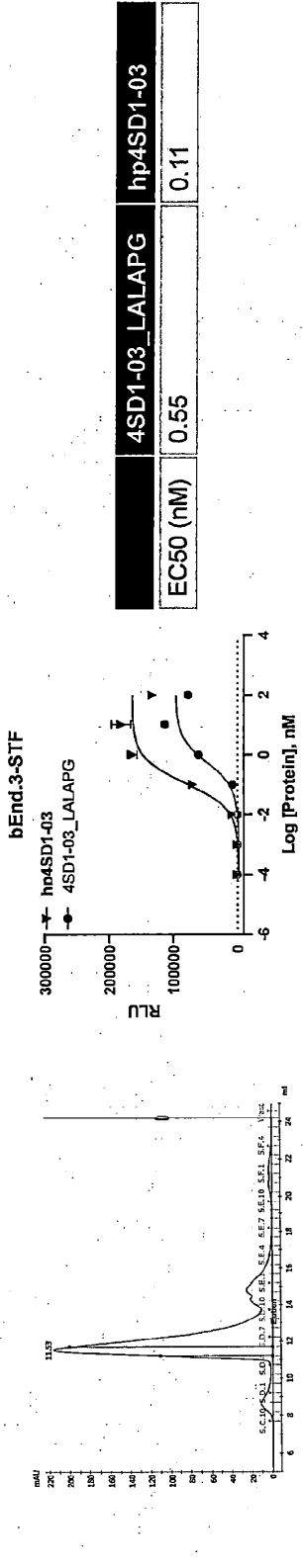
EVQLVESGG-GLVQPGGGSLRLSCAASGFTF...SSVA NSWYRQAP GKGLEWNA ISGS...GGST YVADSVK.G RFTISRDNSK WTLQQLQSLRA EDYATYC AK (SEQ ID NO: 440)

HC

EVQLVESGG-GLVQPGGGSLRLSCAASGFTF...TNYA NSWYRQAP GKGLEWNA ISGS...GGST YVADSVK.G RFTISRDNSK WTLQQLQSLRA EDYATYC ARANGFTWVDFY NGQGTIVTSS (SEQ ID NO: 182)

IGHV4*01
Homo sapiens

YFDY NGQGTIVTSS V (SEQ ID NO: 441)



The framework mutated 4SD1-03_LALAPG retained good expression and STF activity when combined with the polished VHH3 and 4SD1. The polished hp4SD1-03 molecule was more potent than the parental molecule 4SD1-03_LALAPG.

FIG. 8

hp4SD1-03

FIG. 9

LC

EVQLVESGGGLVQPGGSLRLSCAASSANIGSIETLGWYRQAPGCKQRELIA¹NMRGGGMYK²YADSLKGRFIMSTIDNSKNTMYI³
QWNSIRAE⁴EDTAVYGYG⁵YKLRDE⁶DWYRGQGIQVTVSSGGGGSDIOM⁷SSSL⁸SASVGD⁹RVTTICRASQSSSYLNWYQQ¹⁰
KPGKAPKLL¹¹YAGS¹²GGVPSRFGSGG¹³ST¹⁴SSLOPED¹⁵AVY¹⁶GGGK¹⁷VEIK¹⁸RTVAAPSVFIFPPSD¹⁹
EQLKSGTASWVCLLN²⁰IFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS²¹STYSLSSTLTLSKADYEKHKVYACEVTHQGLSS²²
PVTKSFN²³RGE²⁴C (SEQ ID NO:1)

HC

EVQLVESGGGLVQPGGSLRLSCAASSANIGSIETLGWYRQAPGCKQRELIA¹NMRGGGMYK²YADSLKGRFIMSTIDNSKNTMYI³
QWNSIRAE⁴EDTAVYGYG⁵YKLRDE⁶DWYRGQGIQVTVSSGGGGSDIOM⁷SSSL⁸SASVGD⁹RVTTICRASQSSSYLNWYQQ¹⁰
KPGKAPKLL¹¹YAGS¹²GGVPSRFGSGG¹³ST¹⁴SSLOPED¹⁵AVY¹⁶GGGK¹⁷VEIK¹⁸RTVAAPSVFIFPPSD¹⁹
EQLKSGTASWVCLLN²⁰IFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS²¹STYSLSSTLTLSKADYEKHKVYACEVTHQGLSS²²
PVTKSFN²³RGE²⁴C (SEQ ID NO:1)

VHH domain

VL

VH

Fc

Chg. Sequence

CDR: **Black bold font with the underline.**

Gray: LALAPG mutations

Changes from parental (4SD1-03_LALAPG) sequence

Red: Humanization in VHH3

Yellow: Liability de-risking in VHH3 CDRs

Green: Germline humanization in HC framework

Purple: Liability de-risked residues in HC CDRs

hp4SD1-3 (humanized polished) higher efficacy in cell-based assay than the parental 4SD1-03_LALAPG

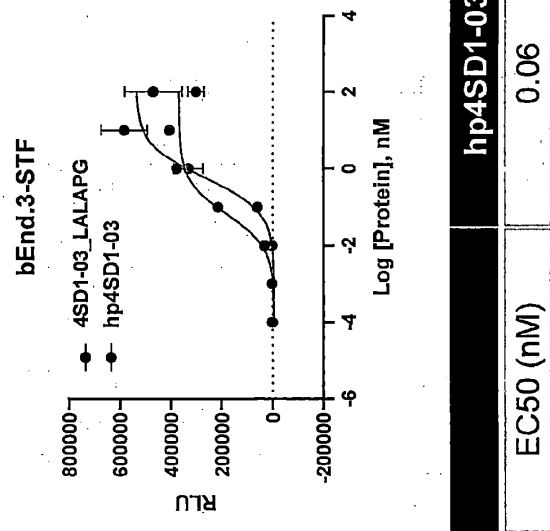
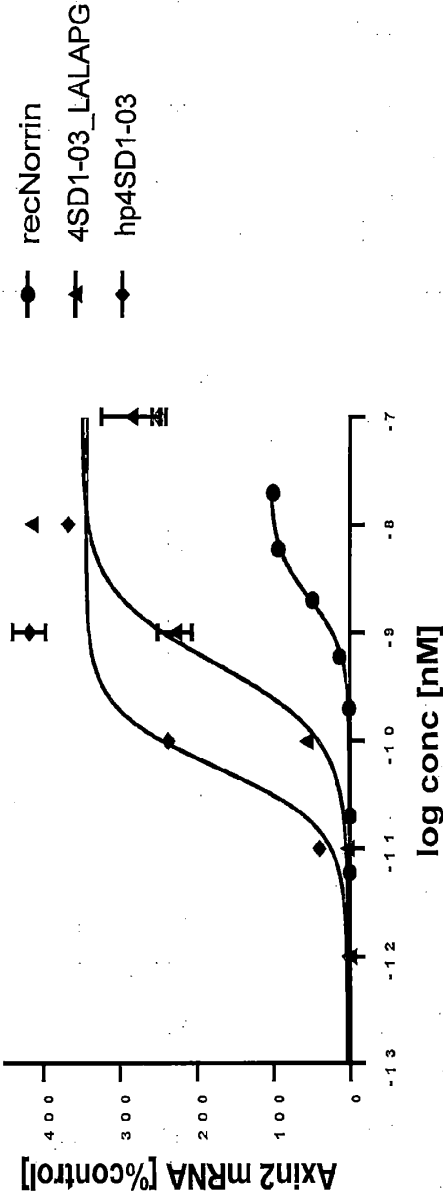


FIG. 10A

hp4SD1-3 (humanized polished) higher efficacy in cell-based assay than the parental 4SD1-03_LALAPG in inducing Axin2 expression representative DRC from treated bEND.3 cells



Average EC₅₀ in nM from 4 independent experiments

	Norrin	4SD1-03_LALAPG	hp4SD1-03
Mean	1.811	1.022	0.08865

FIG. 10B

Mouse OIR_ efficacy comparison between parental and hp4SD1-03

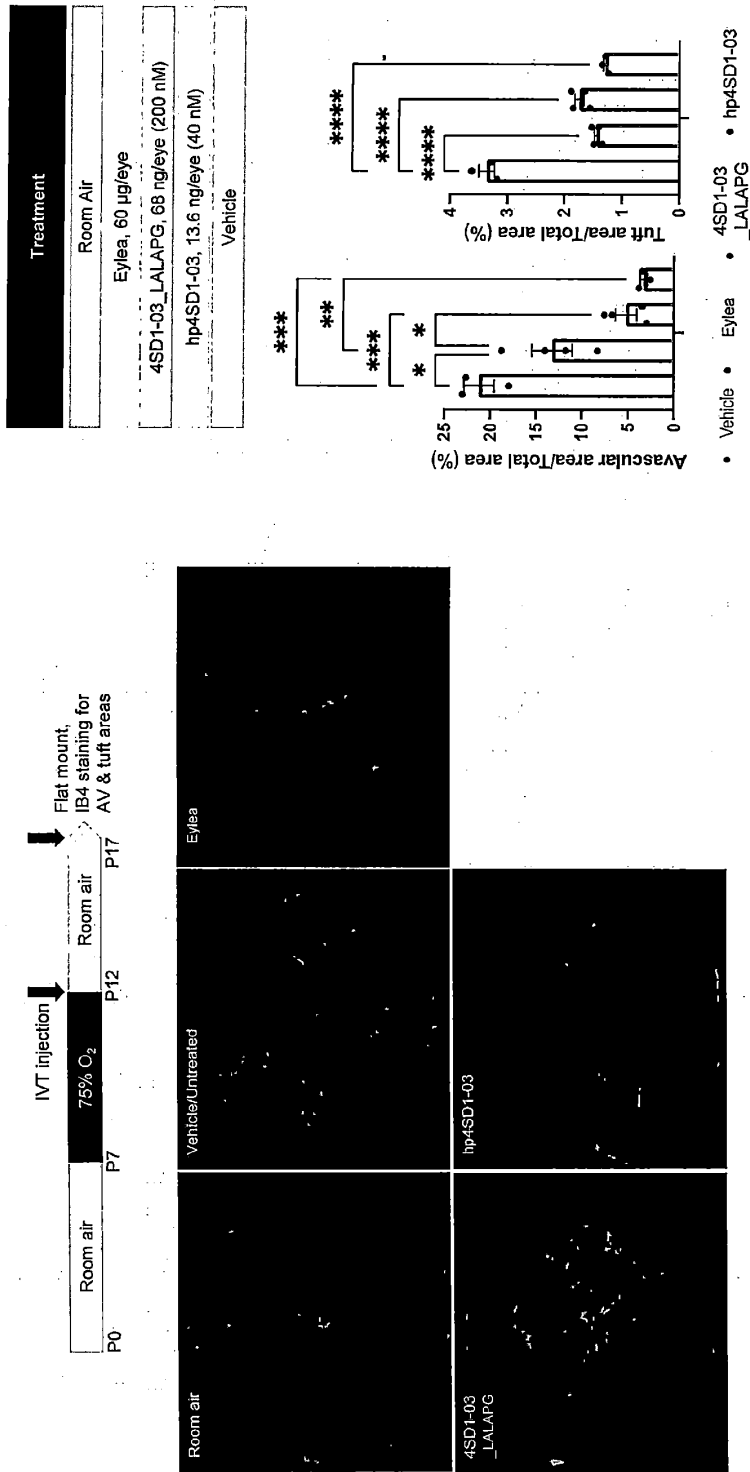
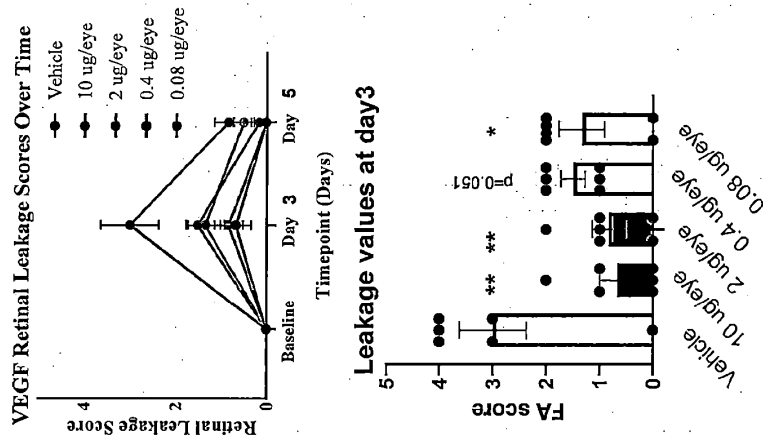


FIG. 11

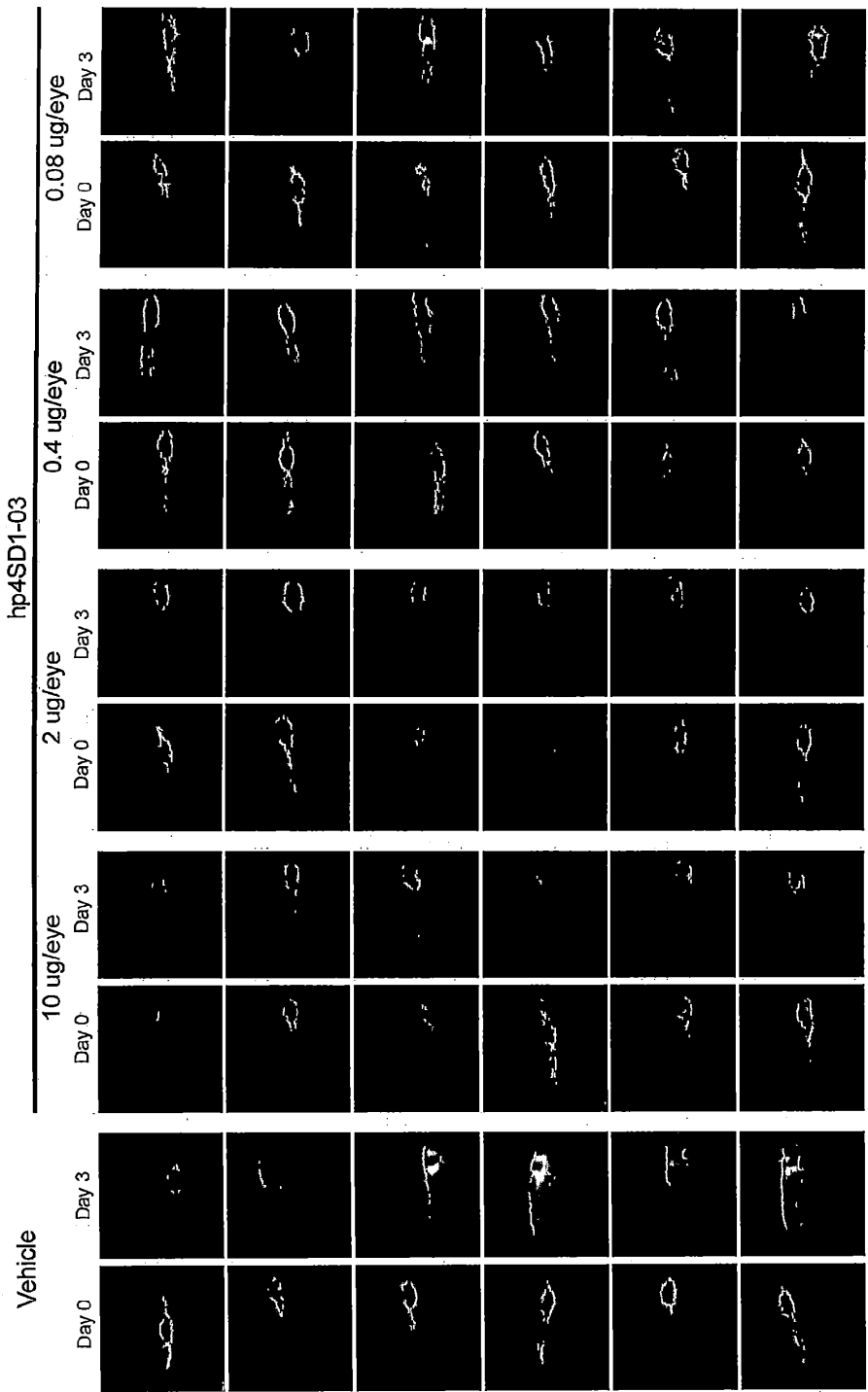
hp4SD1-03 efficacy in VEGF-induced retinal vascular leakage rabbit

Arm	Treatment	No. of animals	Dosing
2	hp4SD1-03_High, 10 ug/eye	6	Day 0
3	hp4SD1-03_Med, 2 ug/eye	6	Day 0
4	hp4SD1-03_Low, 0.4 ug/eye	6	Day 0
5	hp4SD1-03_Low, 0.08 ug/eye	6	Day 0
Totals: 30 rabbits			



hp4SD1-03 shows efficacy in vascular leakage control without any observable ocular abnormalities.

FIG. 12



Retinal vascular leakage comparison between the treatment of vehicle and hp4SD1-03 at Day 3, Efficacy observed in all tested animals

FIG. 13

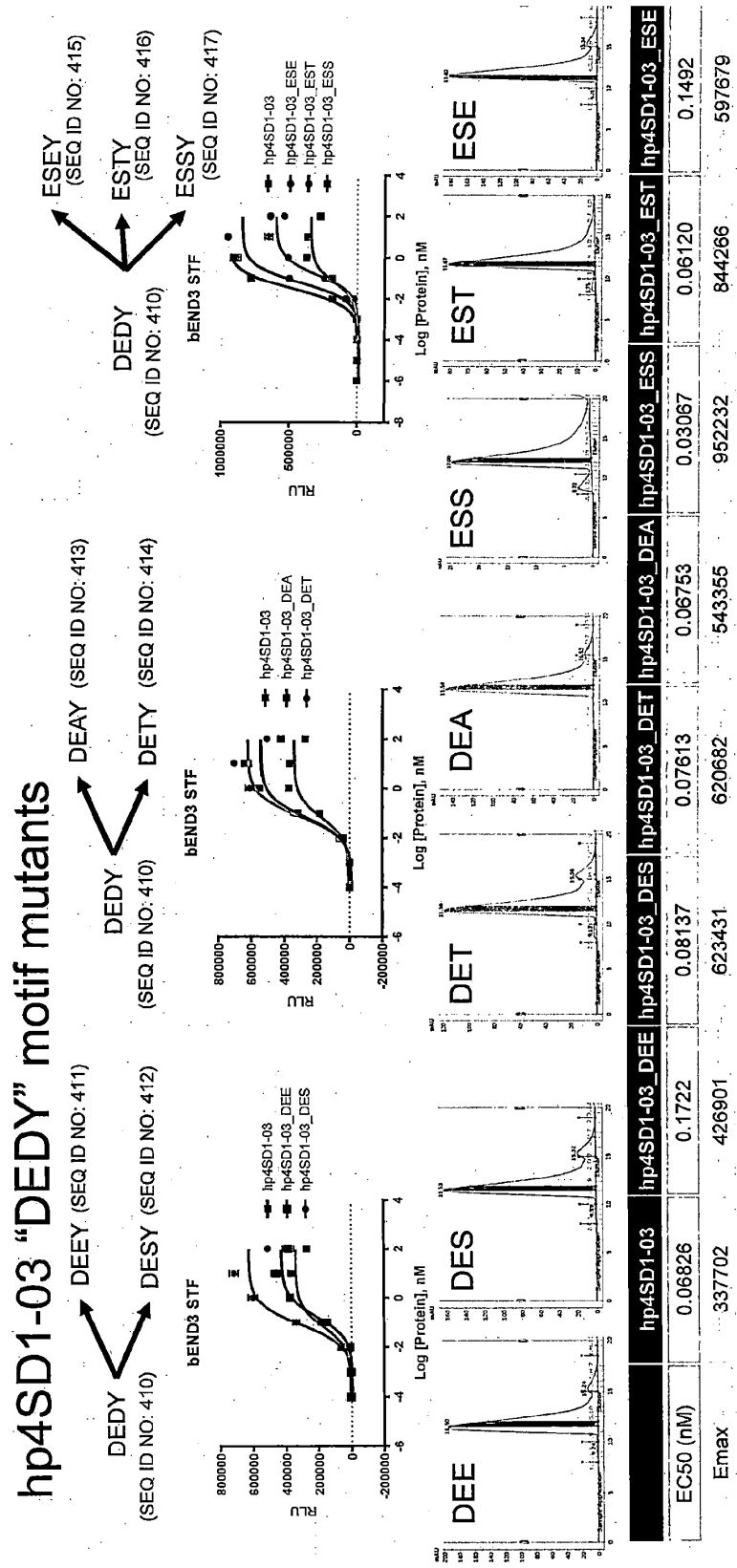


FIG. 14

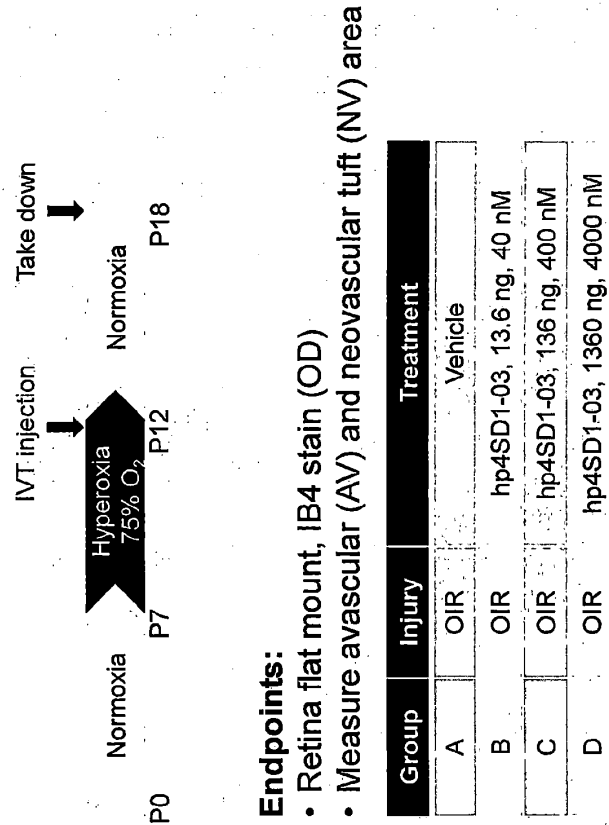


FIG. 15A

Endpoints:

- Retina flat mount, IB4 stain (OD)
- Measure avascular (AV) and neovascular tuft (NV) area

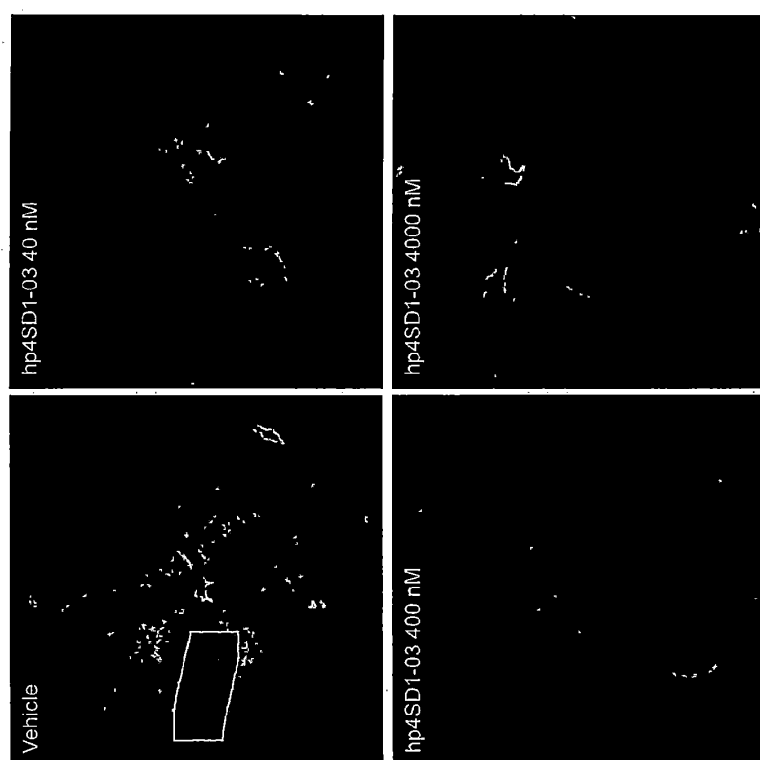


FIG. 15B

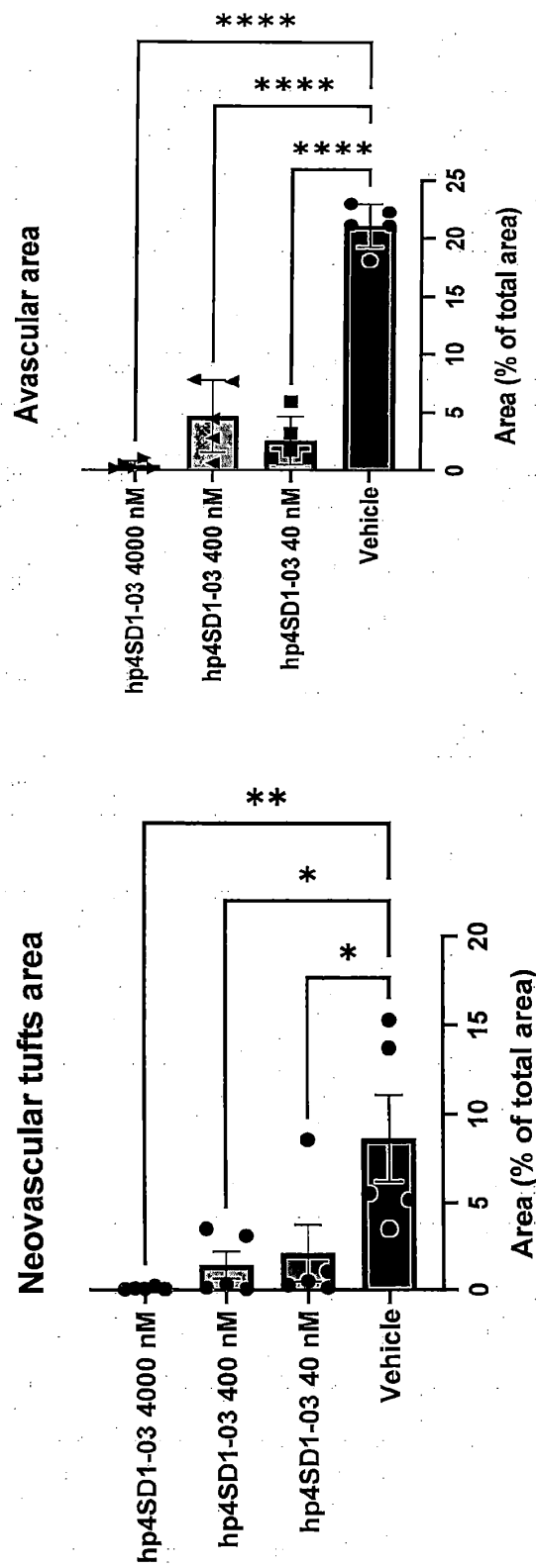


FIG. 15C

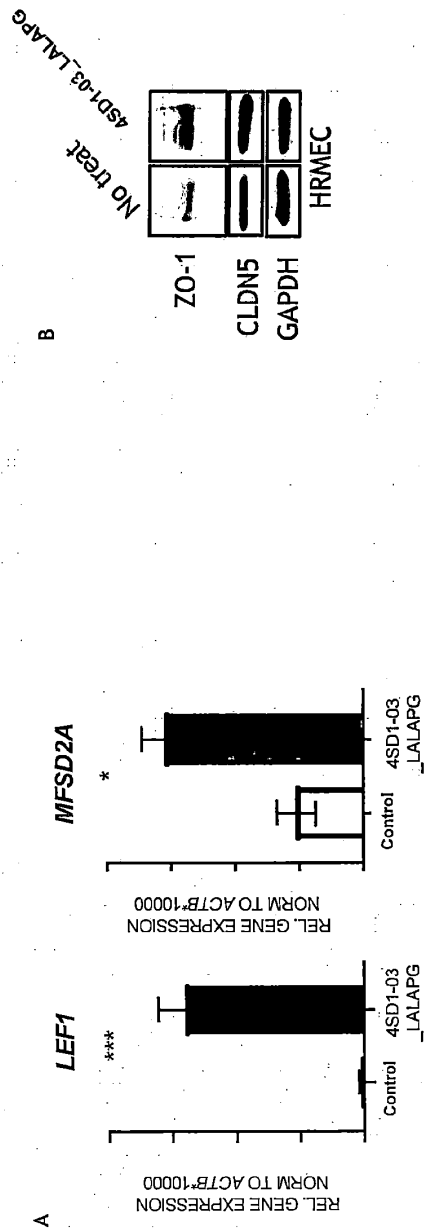
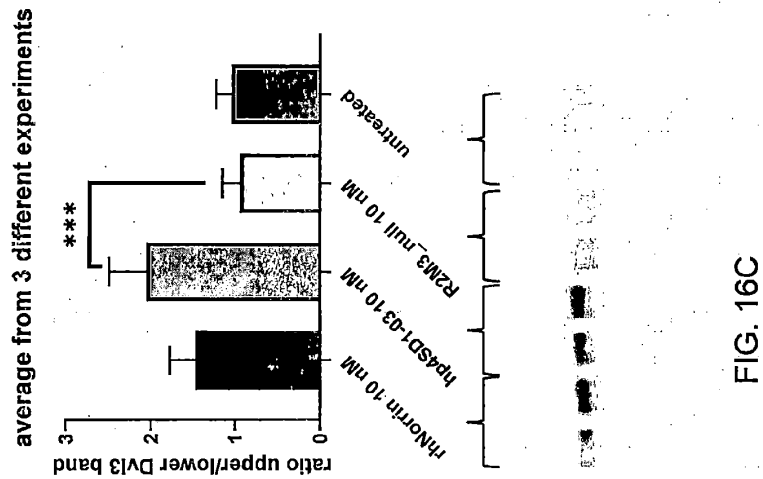


FIG. 16A

FIG. 16B



Increased affinity to Fzd4 CRD (by BLI)

FIG. 17A

4SD1-03_LALAPG

FIG. 17B

hp4SD1-03

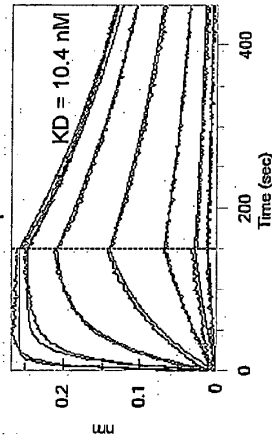
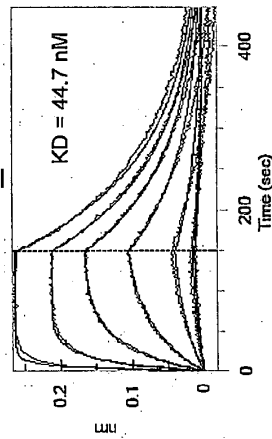


FIG. 17C

Loading Sample	FZD4 CRD Conc. (nM)	KD (M)	kon(1/Ms)	kdis(1/s)
4SD1-03_LALAPG	1000	4.47E-08	2.04E+05	9.13E-03
	333.3	4.47E-08	2.04E+05	9.13E-03
	111.1	4.47E-08	2.04E+05	9.13E-03
	37	4.47E-08	2.04E+05	9.13E-03
	12.4	4.47E-08	2.04E+05	9.13E-03
	4.12	4.47E-08	2.04E+05	9.13E-03
hp4SD1-03	137	4.47E-08	2.23E+05	2.31E-03
	1000	1.04E-08	2.23E+05	2.31E-03
	333.3	1.04E-08	2.23E+05	2.31E-03
	111.1	1.04E-08	2.23E+05	2.31E-03
	37	1.04E-08	2.23E+05	2.31E-03
	12.4	1.04E-08	2.23E+05	2.31E-03

Increased affinity to Fzd4 CRD (by SPR)

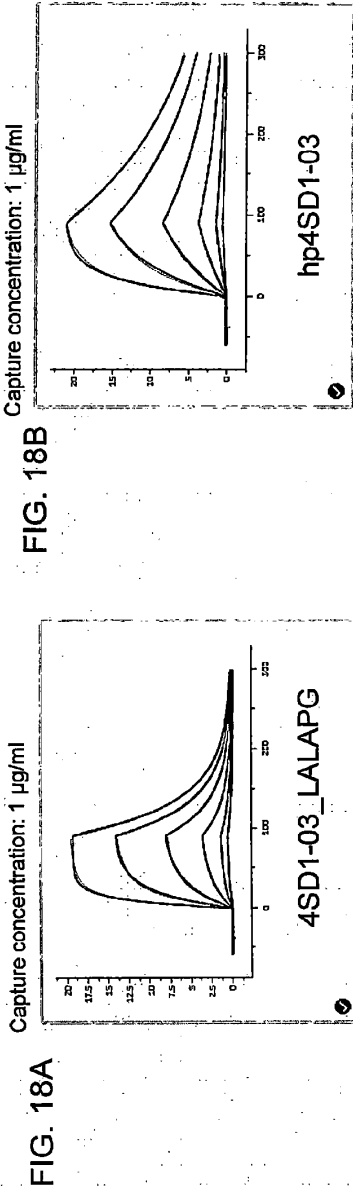


FIG. 18C

Average of measurements at four capture concentrations

Sample	k_a (1/Ms) (std dev)	k_d (1/s) (std dev)	Apparent K_D (M) (std dev)
4SD1-03_LALAPG	3.13E+04 (9.20E+02)	2.28E-02 (9.85E-05)	7.29E-07 (2.17E-08)
hp4SD1-03	1.82E+04 (3.38E+02)	6.28E-03 (3.01E-05)	3.45E-07 (6.34E-09)

hp4SD1-03-AAQ

LC

VHH=Bold; G4S line=light grey; VL= underlined black; constant of Kappa LC = Black Italic

EVQLVESGGGLVQPGGSLRLSCASSANIQSIETLGWYRQAPGKQRELIA~~MM~~RGGGYMKYADSLKG
RFTMSTDNSKNTMYLQMNSLRAEDTAVYYCYVKLRDEDVYRGQGTQVTVSSGGGGSDIQMTQS
PSSLSASVGDRVTITCRASQSISSYLNWYQQKPKGAPKLLIYAASSLQSGVPSRFSGSGGTDFTLTI
SSLQPEDFATYYCQQSYSTPLTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPRE
AKVQWKVDNALQSGNSQESVTEQDSKDSYSTLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSF
NRGEC (SEQ ID NO: 1)

HC

VH= Italic Bold; CH=Black; Fc=Bold underlined

EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYAMSWVRQAPGKGLEWVSAISGSGGGSTYYAESV
KGRFTISRDN~~SKNT~~LYLQMNSLRAEDTAVYYCARATGFGTVFDYWGQGT~~LVTVSS~~ASTKGPSVFP
LAPSSKSTSGGTAALGCLVKDYFPEPTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLG
TQTYICNVNHKPSNTKVDKKVEPKSCDKTH~~TCPPCP~~APAEAGGPSVFLFPPKPKDTLM~~AS~~RTPEVT
CVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL~~AAQ~~DWLN~~NG~~KEYCKKVS
N~~KAL~~GAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN
YKTTTPPVLSDSGSF~~FLYSKLTVDKSRWQQGNVFSCSVMHEALHNQ~~YTQKSLSLSPGK (SEQ ID NO: 450)

FIG. 19A

hp4SD1-03-AAA

LC

VH=Bold; G4S liner=light grey; VL= underlined black; constant of Kappa LC = Black Italic

EVQLVESGGGLVQPGGSLRLSCASSANIQSIETLGWYRQAPGKQRELIANMRGGGYMKYADSLKG
RFTMSTDNSKNTMYLQMNSLRAEDTAVYCYVKLRDEDYVYRGQGTQTVSSGGGSDIQMTQS
PSSLASVGDRTTICRASQSISSYLNWYQQKPKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLT
SSLQPEDFATYYCQQSYSTPLTFGGGKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPRE
AKVQWVKVDNALQSGNSQESVTEQDSKDSYSTYSSLTLSKADYEKHKVYACEVTHQGLSSPVTKSF
NRGEC (SEQ ID NO:1)

HC

VH= Italic Bold; CH=Black; Fc=Bold underlined

EVQLVESGGGLVQPGGSLRLSCAASGFTFTSYAMSWVRQAPGKGLEWVSAISGGGGSTYYAESVK
GRFTISRDN SKNTLYLQMNSLRAEDTAVYCARATGFGTVFDYWGQGT LVTVSSASTKGPSVFPLA
PSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQT
YICNVN HKPSNTKVDKKVEPKSCDKTHCTCPPEAAGGPSVFLFPPKPKDTLMASRTPEVTCVV
DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLAQDWLNGKEYCKKVSNAKLG
APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTP
VLDSGSGFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNAYTQKSLSPGK (SEQ ID NO: 451)

FIG. 19B

hp4SD1-03 and its Fc Mutants hp4SD1-03 AAQ and AAA can be Purified by Protein A Affinity Resin followed by SEC

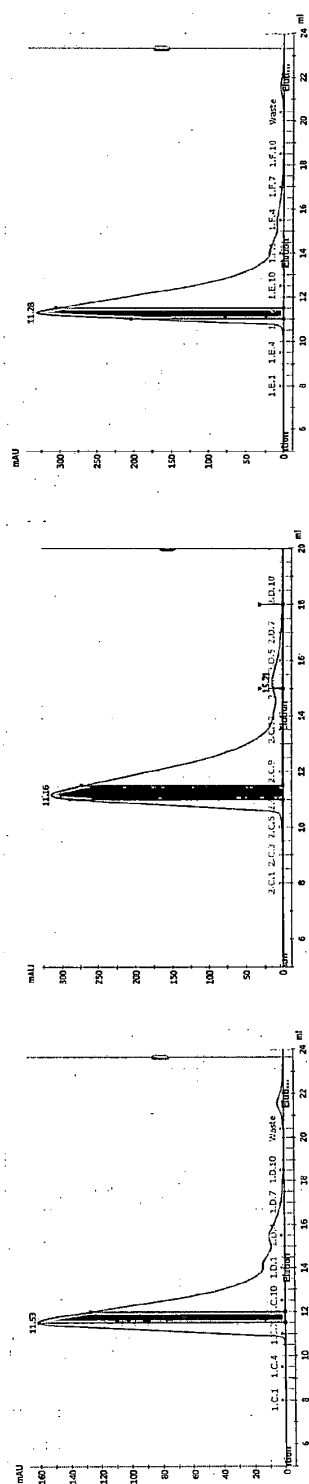


FIG. 20

Melting Temperature of hp4SD1-03 and hp4SD1-03 AAQ and AAA

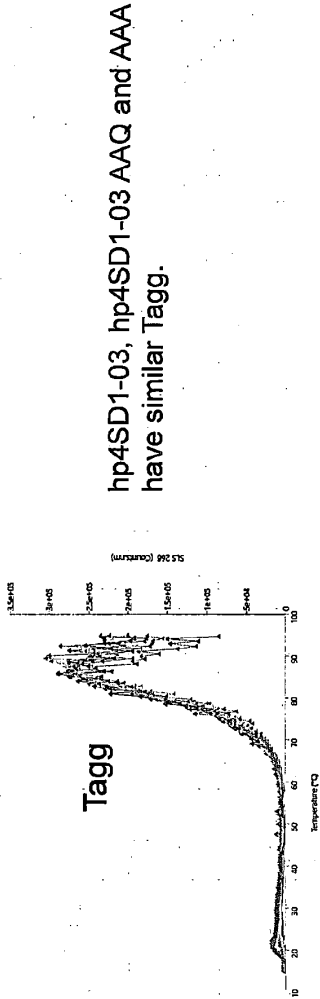
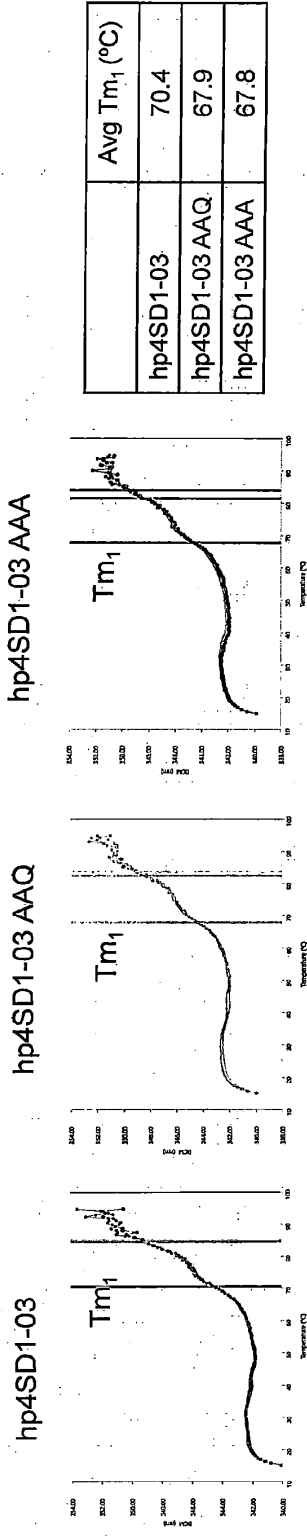


FIG. 21

STF activity comparison: hp4SD1-03 and its Fc Mutants hp4SD1-03-AAQ and AAA

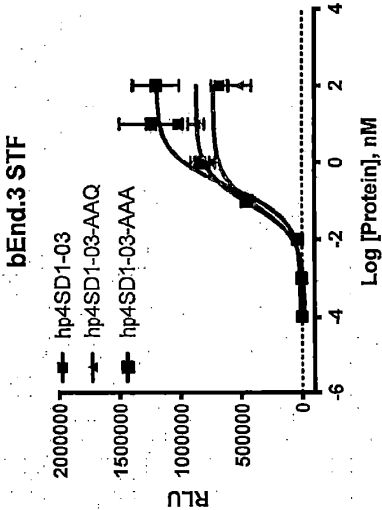
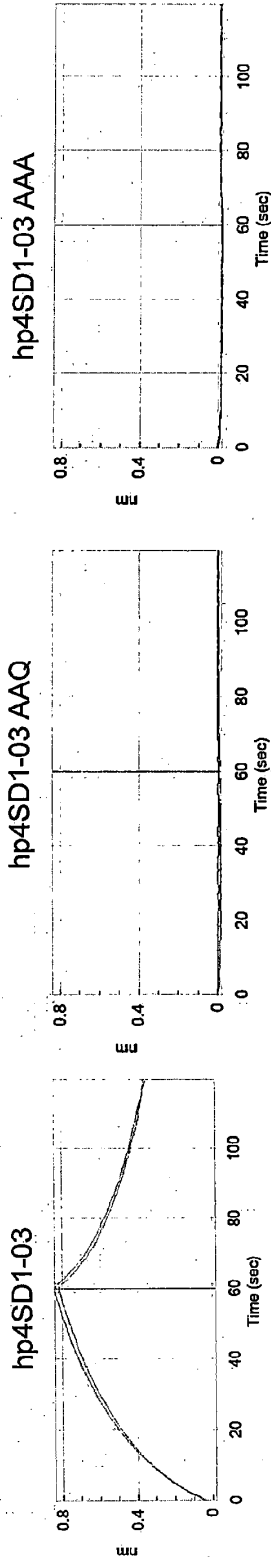


FIG. 22

	hp4SD1-03	hp4SD1-03-AAQ	hp4SD1-03-AAA
EC50 (nM)	0.09	0.07	0.25

Fc Mutants of hp4SD1-03, hp4SD1-03-AAQ and hp4SD1-03-AAA
Does Not Bind to the neonatal Fc-receptor, FcRn



Loading sample: FcRn 100 nM was captured on NTS sensor
Analyte: hp4SD1-03, hp4SD1-03 AAQ, hp4SD1-03 AAA 100 nM

FIG. 23

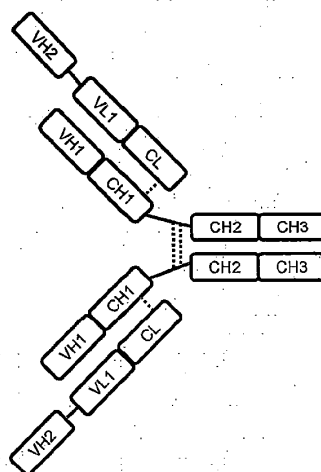
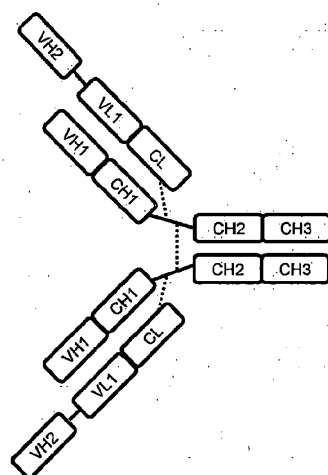
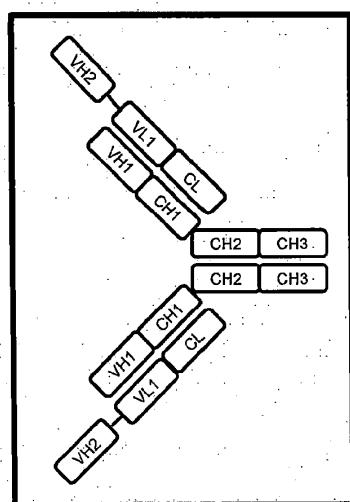


FIG. 24

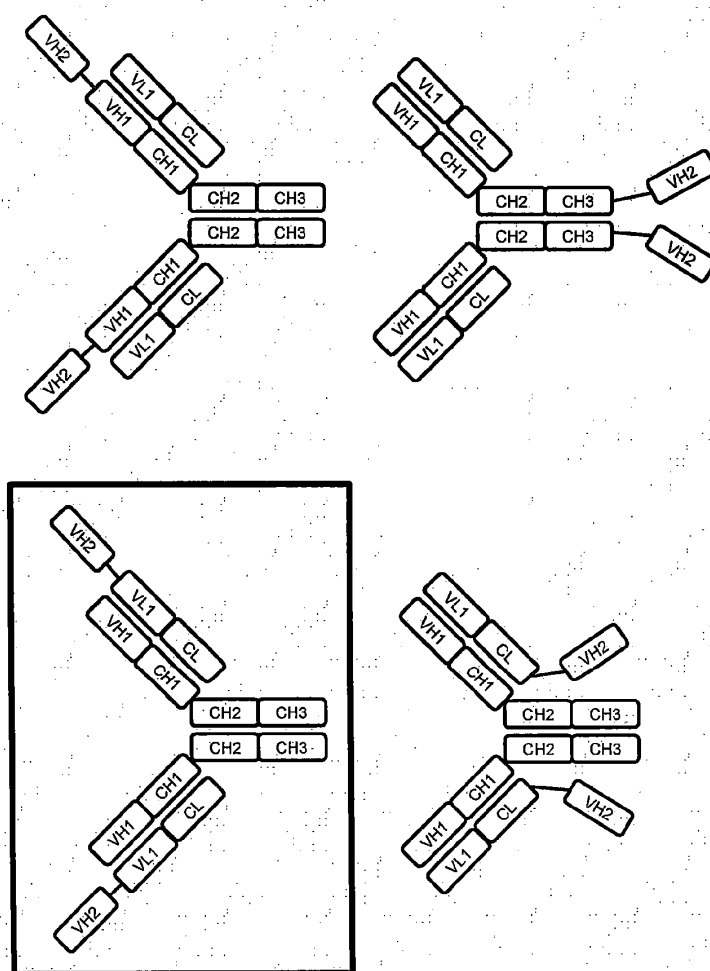


FIG. 25

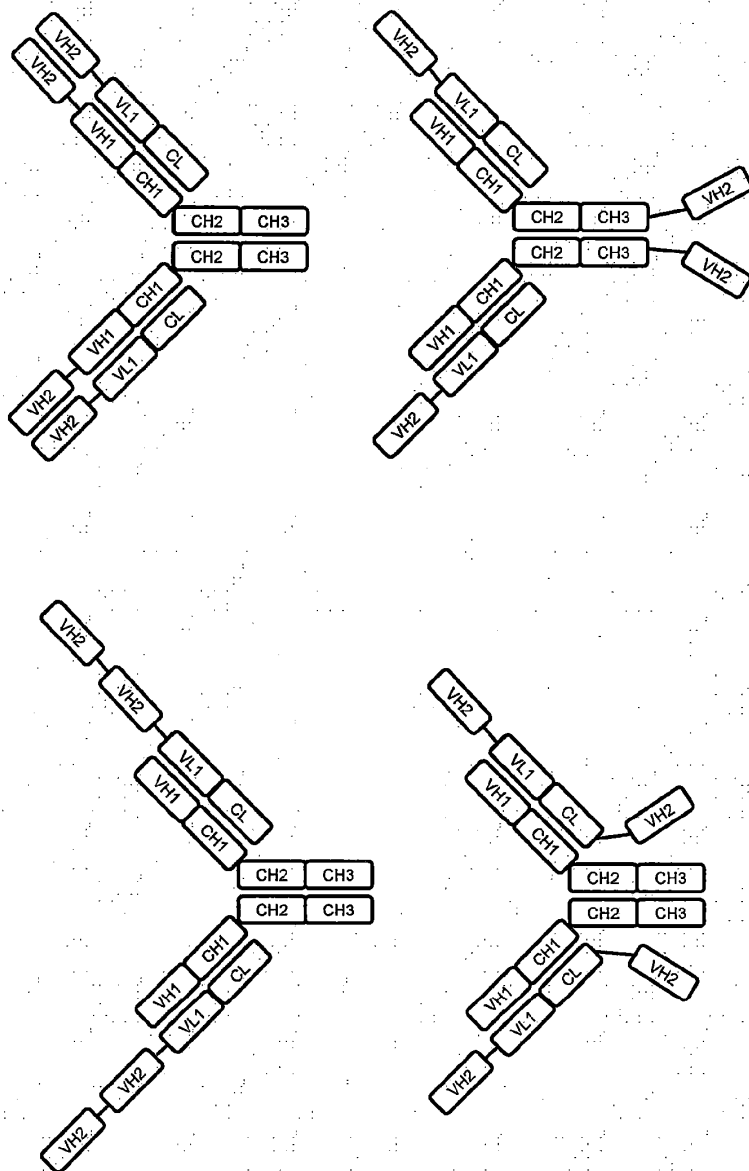


FIG. 26

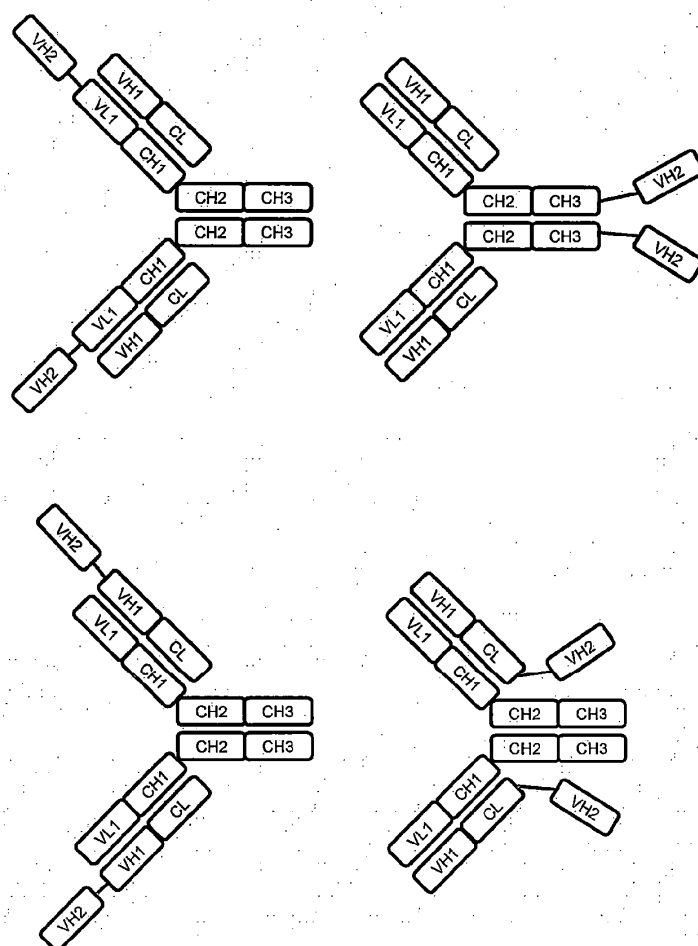


FIG. 27

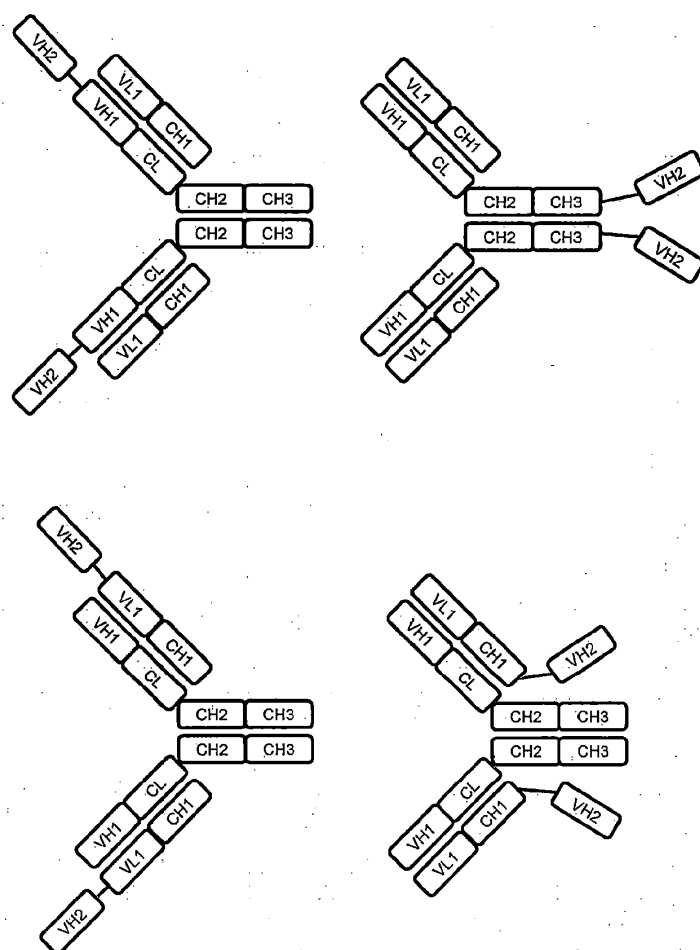


FIG. 28

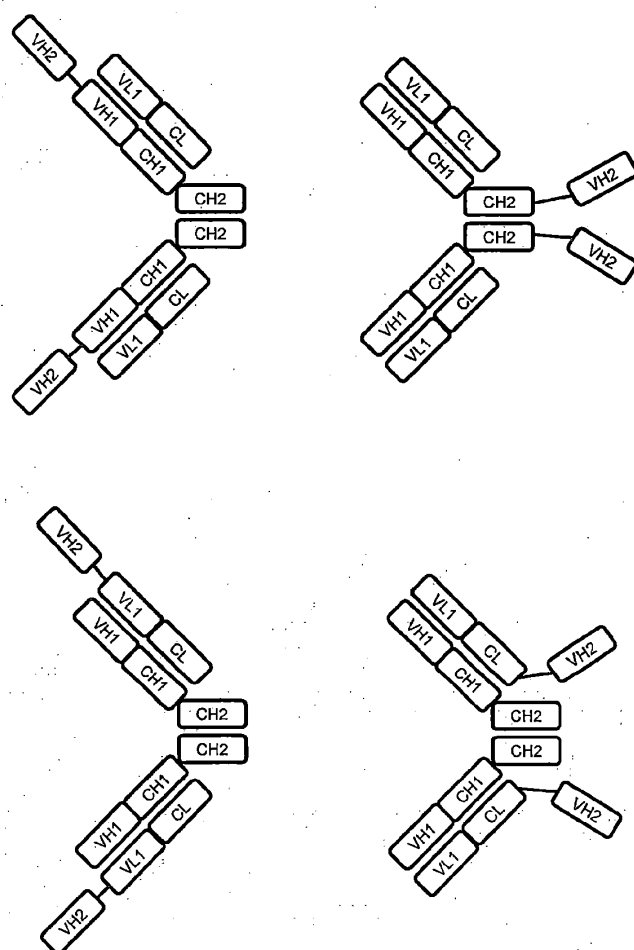


FIG. 29

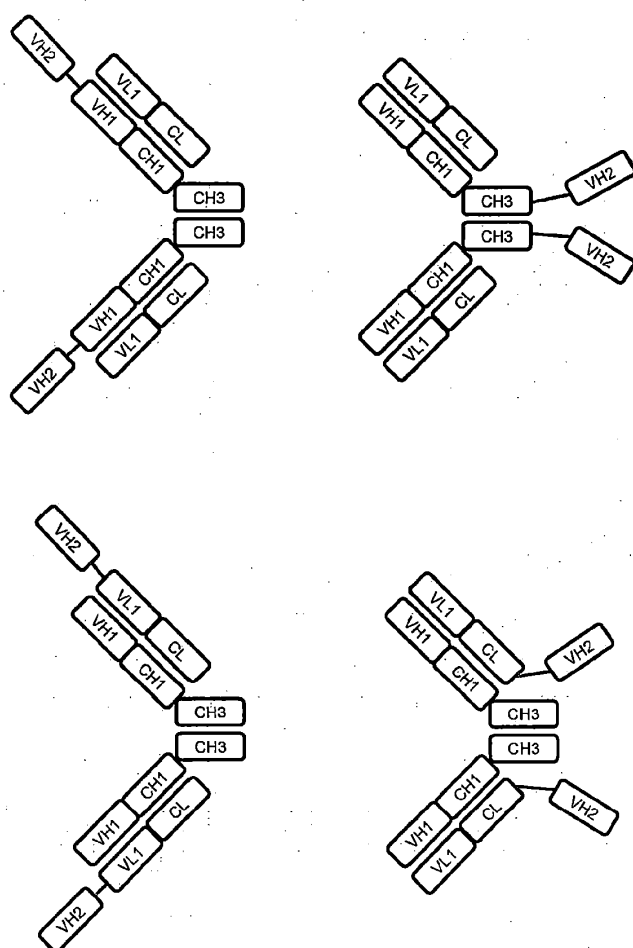


FIG. 30

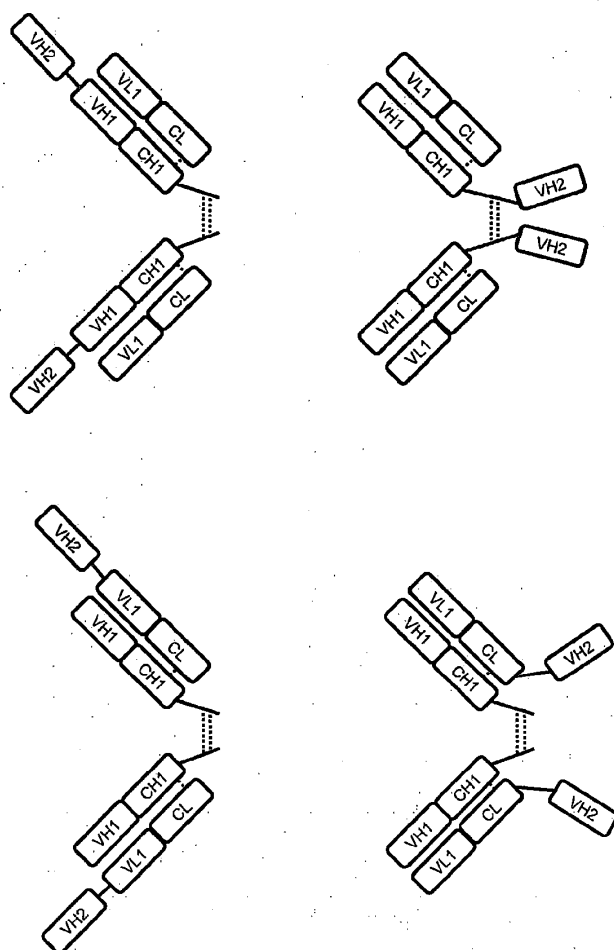


FIG. 31

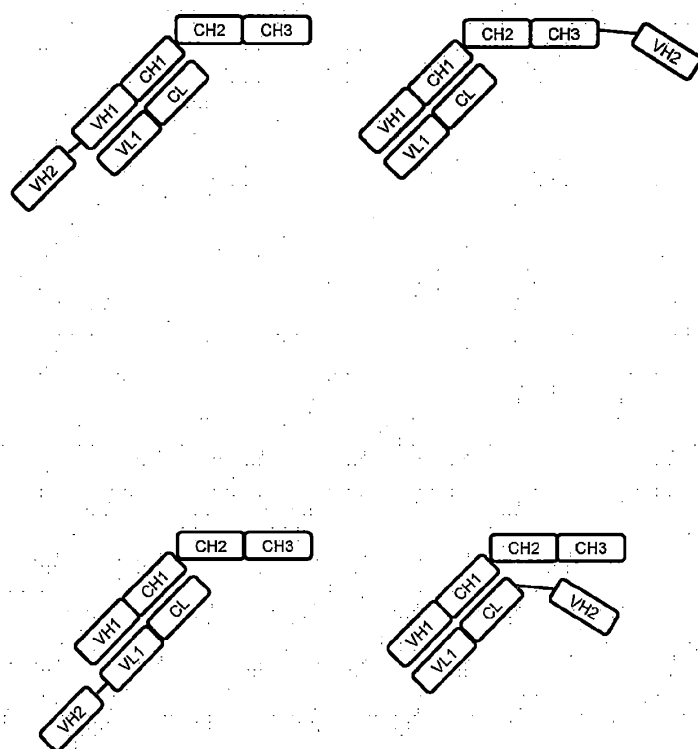


FIG. 32A

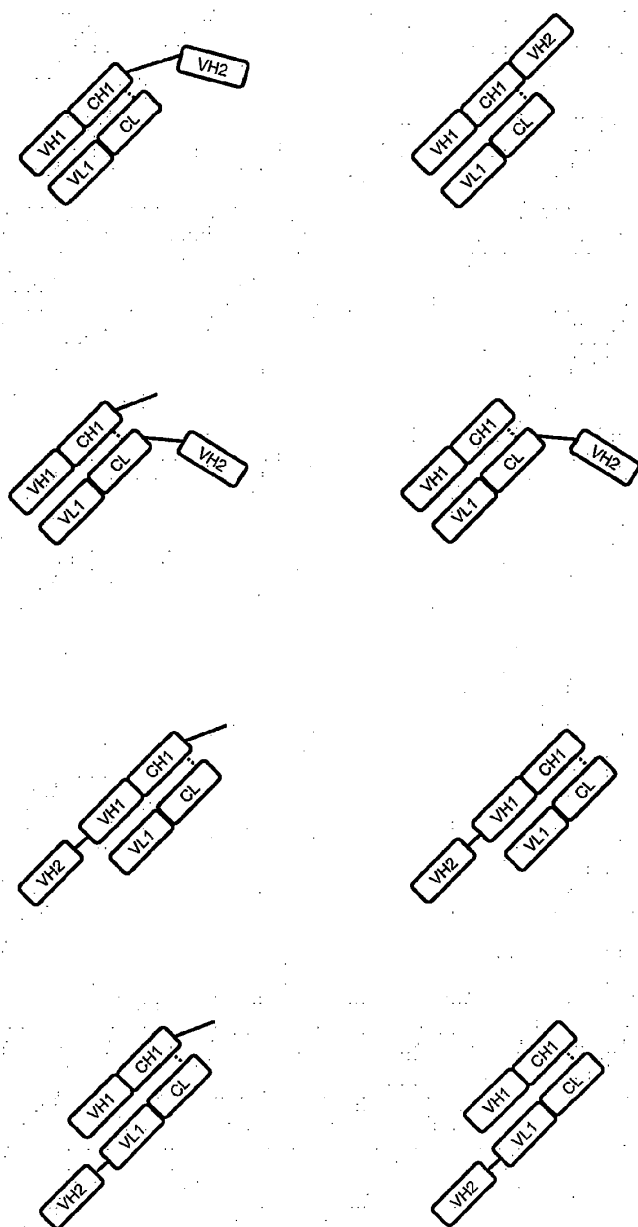


FIG. 32B

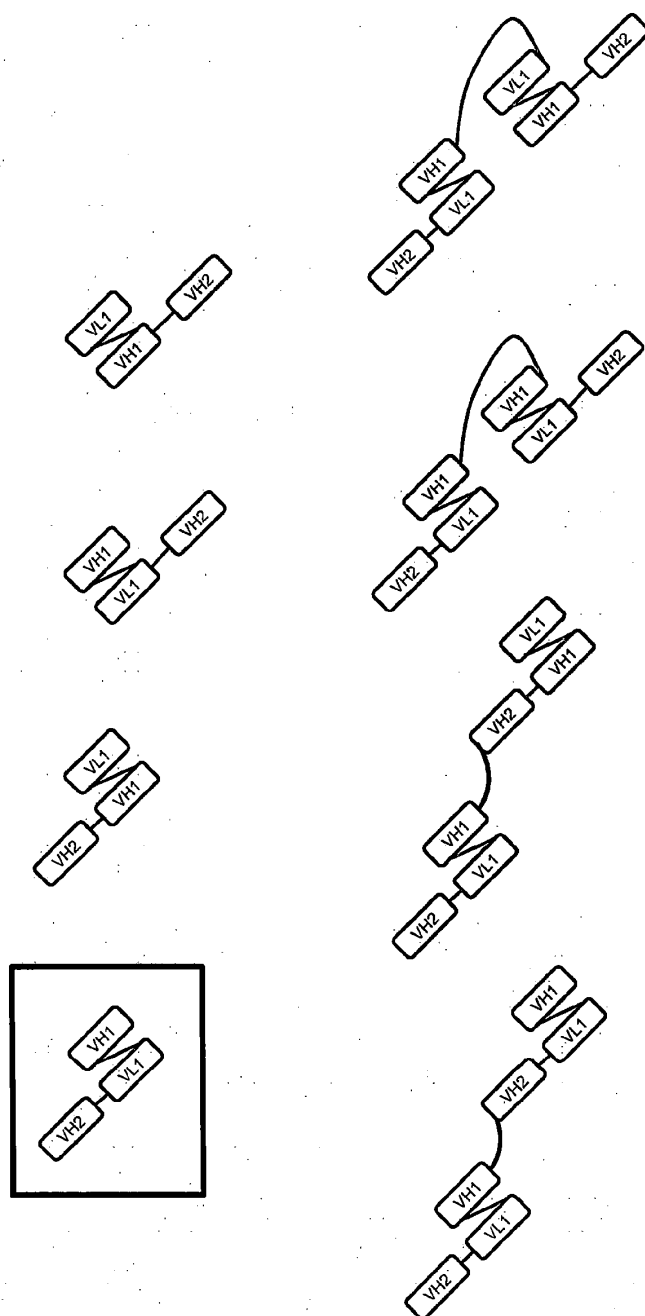


FIG. 33

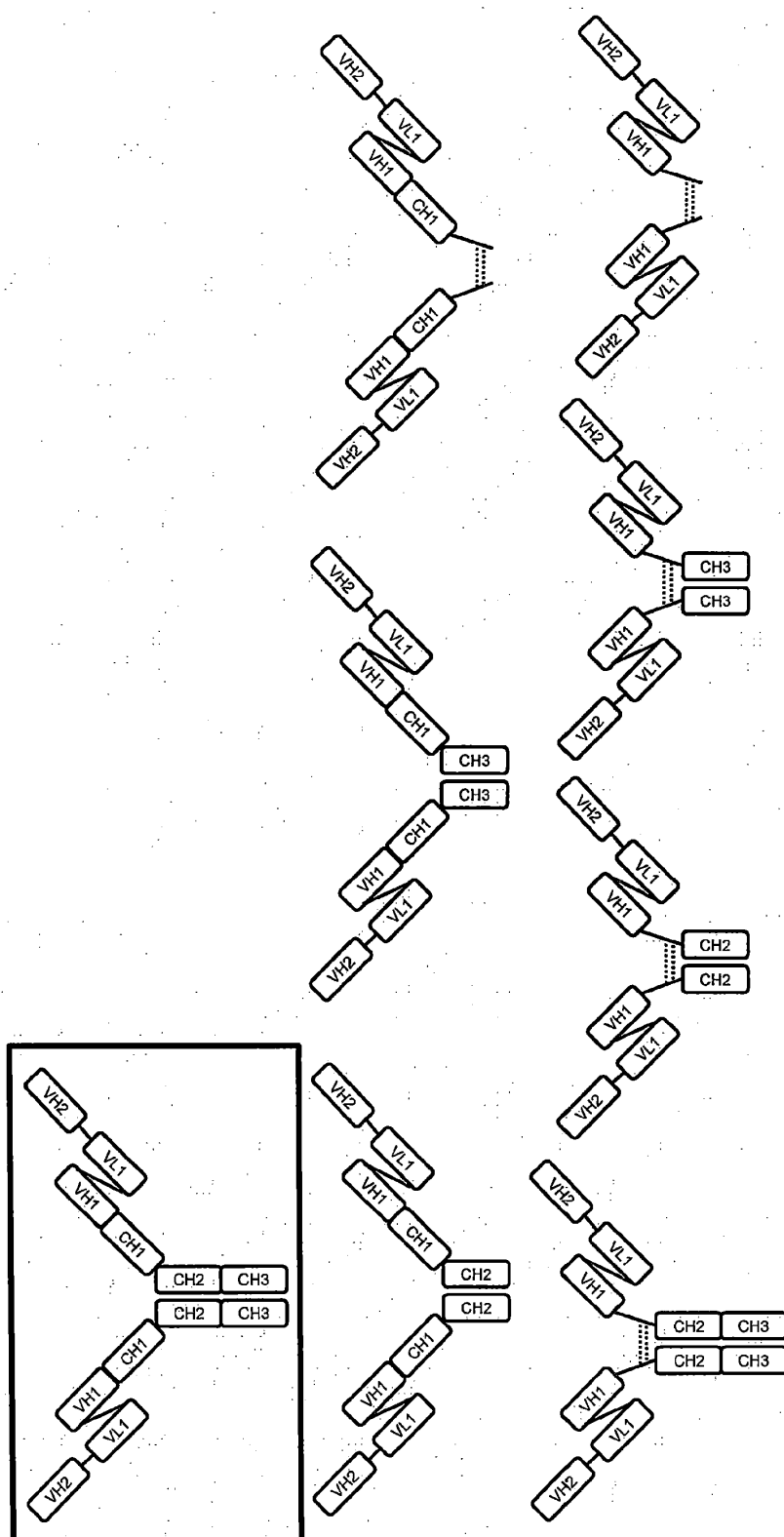


FIG. 34