



(86) **Date de dépôt PCT/PCT Filing Date:** 2009/04/03
(87) **Date publication PCT/PCT Publication Date:** 2009/10/08
(45) **Date de délivrance/Issue Date:** 2016/02/16
(85) **Entrée phase nationale/National Entry:** 2010/09/29
(86) **N° demande PCT/PCT Application No.:** EP 2009/053985
(87) **N° publication PCT/PCT Publication No.:** 2009/121948
(30) **Priorité/Priority:** 2008/04/03 (US61/041,965)

(51) **Cl.Int./Int.Cl. C07K 16/40** (2006.01),
C12N 9/64 (2006.01), **G01N 33/68** (2006.01)

(72) **Inventeurs/Inventors:**
DE STROOPER, BART, BE;
MARJAUX, ELS, BE;
ZHOU, LUJIA, BE;
MUYLDERMANS, SERGE, BE

(73) **Propriétaires/Owners:**
KATHOLIEKE UNIVERSITEIT LEUVEN, K.U.LEUVEN
R&D, BE;
VIB VZW, BE;
VRIJE UNIVERSITEIT BRUSSEL, BE

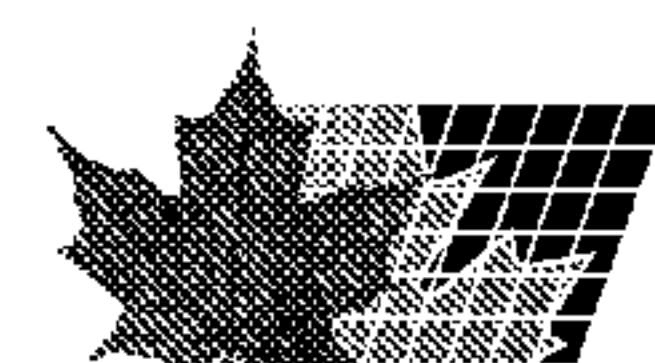
(74) **Agent:** FETHERSTONHAUGH & CO.

(54) **Titre : ANTICORPS A DOMAINE UNIQUE CAPABLES DE MODULER UNE ACTIVITE DE LA BACE**

(54) **Title: SINGLE DOMAIN ANTIBODIES CAPABLE OF MODULATING BACE ACTIVITY**

(57) **Abrégé/Abstract:**

The present invention relates to single domain antibodies with a specificity for BACE1. More specifically, the invention provides single variable domain antibodies derived from camelids which bind to BACE1 and are capable of inhibiting the activity of BACE1. Said antibodies can be used for research and medical applications. Specific applications include the use of BACE1 specific antibodies for the treatment of Alzheimer's disease.



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
8 October 2009 (08.10.2009)

PCT

(10) International Publication Number
WO 2009/121948 A3

(51) International Patent Classification:

C07K 16/40 (2006.01) **G01N 33/68** (2006.01)
C12N 9/64 (2006.01)

(21) International Application Number:

PCT/EP2009/053985

(22) International Filing Date:

3 April 2009 (03.04.2009)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/041,965 3 April 2008 (03.04.2008) US

(71) **Applicants** (for all designated States except US): **VIB VZW** [BE/BE]; Rijnvisschestraat 120, B-9052 Gent (BE). **KATHOLIEKE UNIVERSITEIT LEUVEN, K.U.LEUVEN R&D** [BE/BE]; Minderbroedersstraat 8a - bus 5105, B-3000 Leuven (BE). **VRIJE UNIVERSITEIT BRUSSEL** [BE/BE]; Pleinlaan 2, B-1050 Brussel (BE).

(72) Inventors; and

(75) **Inventors/Applicants** (for US only): **DE STROOPER, Bart** [BE/BE]; Maria Theresiastraat 32, B-3000 Leuven (BE). **MARJAUX, Els** [BE/BE]; Torenstraat 30, B-3384 Attenrode-Wever (BE). **ZHOU, Lujia** [CN/BE]; Parijsstraat 3, Studio 24, B-3000 Leuven (BE). **MUYLDERMANS, Serge** [BE/BE]; Klein Vuurgatstraat 3, B-1560 Hoeilaart (BE).

(74) **Common Representative:** **VIB VZW**; Rijnvisschestraat 120, B-9052 Gent (BE).

(81) **Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(88) Date of publication of the international search report:

7 January 2010

(54) **Title:** SINGLE DOMAIN ANTIBODIES CAPABLE OF MODULATING BACE ACTIVITY

(57) **Abstract:** The present invention relates to single domain antibodies with a specificity for BACE1. More specifically, the invention provides single variable domain antibodies derived from camelids which bind to BACE1 and are capable of inhibiting the activity of BACE1. Said antibodies can be used for research and medical applications. Specific applications include the use of BACE1 specific antibodies for the treatment of Alzheimer's disease.



WO 2009/121948 A3

Single domain antibodies capable of modulating BACE activityField of the invention

The present invention relates to single domain antibodies with a specificity for BACE1. More specifically, the invention provides single variable domain antibodies derived from camelids which bind to BACE1 and are capable of inhibiting the activity of BACE1. Said antibodies can be used for research and medical applications. Specific applications include the use of BACE1 specific antibodies for the treatment of Alzheimer's disease.

Background of the invention

Alzheimer's disease ("AD") is a devastating neurodegenerative disease that affects millions of elderly patients worldwide and is the most common cause of nursing home admittance. AD is characterized clinically by progressive loss of memory, orientation, cognitive function, judgment and emotional stability. With increasing age, the risk of developing AD increases exponentially, so that by age 85 some 20-40% of the population is affected. Memory and cognitive function deteriorate rapidly within the first 5 years after diagnosis of mild to moderate impairment, and death due to disease complications is an inevitable outcome. Definitive diagnosis of AD can only be made post-mortem, based on histopathological examination of brain tissue from the patient. Two histological hallmarks of AD are the occurrence of neurofibrillar tangles of hyperphosphorylated tau protein and of proteinaceous amyloid plaques, both within the cerebral cortex of AD patients. The amyloid plaques are composed mainly of a peptide of 37 to 43 amino acids designated beta-amyloid, also referred to as beta-amyloid, amyloid beta or Abeta. It is now clear that the Abeta peptide is derived from a type 1 integral membrane protein, termed beta amyloid precursor protein (also referred to as APP) through two sequential proteolytic events. First, the APP is hydrolyzed at a site N-terminal of the transmembrane alpha helix by a specific proteolytic enzyme referred to as beta-secretase (the membrane-bound protease BACE1). The soluble N-terminal product of this cleavage event diffuses away from the membrane, leaving behind the membrane-associated C-terminal cleavage product, referred to as C99. The protein C99 is then further hydrolyzed within the transmembrane alpha helix by a specific proteolytic enzyme referred to as gamma-secretase. This second cleavage event liberates the Abeta peptide and leaves a membrane-associated "stub". The Abeta peptide thus generated is secreted from the cell into the extracellular matrix where it eventually forms the amyloid plaques associated with AD. Despite intensive research during the last 100 years, prognosis of AD patients now is still quite the same as that of patients a century ago, since there is still no real cure available. There are two types of drugs

approved by the U.S. Food and Drug Administration and used in clinic today to treat AD: Acetylcholinesterase (AChE) inhibitors and Memantine. There is ample evidence in the art that the amyloid beta peptide, the main component of the amyloid plaques that are specific to the AD etiology, has a key role in the development of AD disease. Therefore, one of the most favourite strategies to lower A β is to diminish its production by γ - and β -secretase inhibitors. One strategy was the development of gamma-secretase inhibitors however such inhibitors often result in serious side effects since gamma-secretase is involved in the proteolytic processing of at least 30 proteins. Yet another attractive strategy is the development of BACE1 inhibitors. BACE1 is produced as a prepropeptide. The 21 amino acids signal peptide translocates the protease into the ER where the signal peptide is cleaved off and from where BACE1 is then directed to the cell surface. After its passage through the trans-Golgi network (TGN), part of BACE1 is targeted to the cell surface from where it is internalized into early endosomal compartments. BACE1 then either enters a direct recycling route to the cell surface or is targeted to late endosomal vesicles destined for the lysosomes or for the TGN. At the TGN it might be retransported to the cell membrane. Given its long half-life and fast recycling rate, mature BACE1 may cycle multiple times between cell surface, endosomal system and TGN during the course of its lifespan. BACE1 inhibitory antibodies are described in US20060034848. In the present invention we sought to develop alternative inhibitors of the activity of BACE1 through the generation of single chain antibodies with a specificity for BACE1. In the resulting collection of binders of BACE1 we identified inhibitors of BACE1. In particular, these BACE1 specific camelid antibodies capable of inhibiting BACE1 activity can be used for the treatment of Alzheimer's disease.

Figures

Figure 1: Amino acid sequence alignment of the variable domain of the BACE1-specific dromedary HCAbs (SEQ ID NOs: 1-14 and SEQ ID NOs: 38-43). V_HH hallmark residues (F/Y₃₇, E/Q₄₄, R₄₅ and G₄₇) are indicated in bold, whereas residues characteristic for a V_H-motif (L₁₁, V₃₇, G₄₄, L₄₅ and W₄₇) are labelled in italics. Cysteine residues other than the canonical C₂₂ and C₉₂ are underlined. Numbering and grouping of residues into either framework or CDR regions are as defined by Kabat (Kabat et al., 1991).

Figure 2: Capacity of the different BACE1 binders to recognize their antigen. **A.** A RaPID plot representing the kinetic rate values k_{on} (M⁻¹s⁻¹) and k_{off} (s⁻¹) for the nanobody-immunogen interactions as determined by surface plasmon resonance (BIAcore). The ratio of k_{off} to k_{on} gives the dissociation constant or K_D . Kinetic constants were measured at pH7.0

(black spots) and pH5.0 (red spots). The majority of the BACE1 binders has K_D values between 10 and 100 nM at both pH conditions.

B. Capacity of the different BACE1 binders to pull down BACE1 from cell lysates. Lysates of BACE1-transfected COS cells were incubated with equal amounts (2 μ g) of the various recombinant his-tagged anti-BACE1 nanobodies. Following pull down of the nanobodies, samples were subjected to SDS-PAGE and analyzed by Western Blotting using anti-BACE1 (ProSci). Nb_BCIIIP and Nb_A β 3, raised against beta-lactamase BCII 569/H (Conrath et al., 2001a) and A β peptide respectively, were used as negative controls. Only part of the nanobodies, raised against non-glycosylated BACE1 ectodomain, are able to efficiently capture glycosylated BACE1 from COS cell lysates.

Figure 3: Effect of Nb_B26 and Nb_B9 on APP processing in cells.

A. Schematic representation of the cDNA construct used to express V_HHs into mammalian cells. The construct consists of the V_HH cDNA, fused at its C-terminus to the signal peptide of BACE1, to ensure ER translocation, and at its C-terminus it is fused to a myc-epitope tag.

B. Nb_B9, but not Nb_B26, adversely affects β -site APP processing upon transient overexpression. COS-B1 cells, stably expressing low levels of BACE1, were co-transfected with APP_{Sw} and either Nb_B26 or Nb_B9. Control cells were either transfected with empty vector or with APP_{Sw} alone. Two days after transfection, cells were lysed and total protein extracts were analyzed by Western blotting using anti-myc, anti-BACE1 (ProSci) and B63.1, to detect V_HHs, BACE1 and APP full length and CTFs (C83 and C99), respectively. One representative experiment is shown.

C. Western blots as the one shown in **C** were probed with GARIR800, an infrared-coupled secondary antibody, and then scanned on an Odyssey scanner. The signal intensity of the APP CTFs was quantified using the Odyssey Application Software v1.2.15 (LI-COR). The ratio of β -CTF to total CTFs (mean $\pm$ SEM, n = 8 to 10), normalized to the ratio of non-transfected cells (set as 1), shows that Nb_B9 could consistently decrease activity by about 30% (*t*-test, *p* < 0,001), whereas Nb_B26 had no impact on APP processing.

Figure 4: V_HH Nb_B9 inhibits β -secretase cleavage of APP by adding to the medium of cultured cells. Neuroblastoma cells SH-SY5Y/ APPwt were treated with 3 μ M Nanobodies for 24 hours, sAPP α and sAPP β from conditioned medium were analyzed by Western blot. Cells treated with nanobody B9 (SEQ ID NO: 6) showed a significant decrease in sAPP β producing.

Figure 5. Amino acid sequence alignment of BACE1-specific V_HHs isolated from dromedary and llama V_HH libraries. Numbering and grouping of residues into either framework or CDR regions are as defined by Kabat (Kabat et al.,1991).

5 **Figure 6.** Western blot analysis of sAPP β and sAPP α from conditioned medium of neuroblastoma cells SH-SY5Y/APPwt treated with nanobodies by adding to the medium at final concentration of 20 μ M. Cells treated with nanobody B9 (SEQ ID NO: 6), 10C4 (SEQ ID NO: 22) and 4A2 (SEQ ID NO: 26) showed a significant decrease in sAPP β producing.

10 **Figure 7.** The inhibition effects of different nanobodies (10 μ M) on BACE1 activity in FRET assay at a concentration of 10 μ M. In this cell-free enzymatic assay, Nb_B9 (SEQ ID NO: 6), Nb_10C4 (SEQ ID NO: 22), Nb_4A2 (SEQ ID NO: 26) and Nb_1B3 (SEQ ID NO: 15) significantly modulate BACE1 activity.

15 **Figure 8.** Dose-response curve of nanobodies 10C4 (**A**), 4A2 (**B**) and B9 (**C**) on BACE1 cleavage activity in FRET assay using a small peptide substrate. Nb_10C4 and Nb_4A2 significantly inhibit BACE1 activity. Nb_B9 significantly increases BACE1 activity.

20 **Figure 9.** Dose-response curve of Nb_B9 on BACE1 cleavage activity in MBP-ELISA using a big peptide substrate. In this cell-free enzymatic assay, Nb_B9 significantly inhibits BACE1 activity.

25 **Figure 10.** V_HHs Nb_B9 (SEQ ID NO: 6) and Nb_4A2 (SEQ ID NO: 26) inhibit BACE1 cleavage of APPwt in primary cultured mouse neurons, as reflected in a decrease of A β , sAPP β and CTF β . Primary cultured neurons from wild type mice were transduced with APPwt by Semliki Forest Virus (SFV), and then treated with purified Nb_B9 and Nb_4A2 by adding to the medium at a final concentration of 20 μ M (V_HHs were first dissolved in PBS), neurons treated with PBS were used as a negative control. After 16-hours treatment, conditioned medium and cell extract were analyzed by Western blot for APP-FL, CTF β , CTF α , A β , sAPP β and sAPP α / β .
30

35 **Figure 11.** Dose response curve of V_HHs Nb_B9 (SEQ ID NO: 6) inhibiting BACE1 in primary cultured mouse neurons established by metabolic labeling assays after 6 hours treatment. Primary cultured neurons from wild type mice were transduced with APPwt by Semliki Forest Virus (SFV), and treated with purified V_HH B9 by adding to the medium serial dilutions (V_HH B9

was first dissolved and diluted in PBS). Neuron cultures were metabolic labeled for 6 hours, APP-FL and CTF β from cell extracts were analyzed by phosphorimaging, while sAPP β , A β and sAPP α from conditioned medium were analyzed by Western Blot.

5

Aims and detailed description of the invention

The present invention relates to BACE1 single variable domain antibodies which can be used in research and medical applications. More specifically, the invention relates to the detection of BACE1 overexpression and to the treatment of Alzheimer's disease using BACE1 single domain antibodies. In the present invention the antibodies are devoid of any light chain but
10 comprise at least one heavy chain antibody. In a particular embodiment the variable domain of a heavy chain antibody is derived from camelids. Such a variable domain heavy chain antibody is herein designated as a Nanobody or a V_HH antibody. Nanobody(TM), Nanobodies(TM) and Nanoclone(TM) are trademarks of Ablynx NV (Belgium).

15 Thus in a first embodiment the invention provides a single variable domain antibody, devoid of a light chain, specifically binding to BACE1. In a particular embodiment said single domain antibody is derived from camelids. In the family of 'camelids' immunoglobulins devoid of light polypeptide chains are found. "Camelids" comprise old world camelids (*Camelus bactrianus* and *Camelus dromaderius*) and new world camelids (for example *Lama paccos*, *Lama glama* and *Lama vicugna*).
20

In another embodiment the invention provides a single domain antibody derived from camelids which amino acid sequence comprises SEQ ID NOs: 1-28. The amino acid sequences of the dromedary/llama Nanobodies (also designated as V_HH antibodies) are depicted in Figures 1 and 5. Nanobody B1 (Nb_B1) corresponds with SEQ ID NO: 1, Nanobody B2 (Nb_B2)
25 corresponds with SEQ ID NO: 2, Nanobody B3 (Nb_B3) corresponds with SEQ ID NO: 3, Nanobody B5 (Nb_B5) corresponds with SEQ ID NO: 4, Nanobody B8 (Nb_B8) corresponds with SEQ ID NO: 5, Nanobody B9 (Nb_B9) corresponds with SEQ ID NO: 6, Nanobody B10 (Nb_B10) corresponds with SEQ ID NO: 7, Nanobody 11 (Nb_B11) corresponds with SEQ ID NO: 8, Nanobody 12 (Nb_B12) corresponds with SEQ ID NO: 9, Nanobody 15 (Nb_B15) corresponds with SEQ ID NO: 10, Nanobody 16 (Nb_B16) corresponds with SEQ ID NO: 11,
30 Nanobody 21 (Nb_B21) corresponds with SEQ ID NO: 12, Nanobody 25 (Nb_B25) corresponds with SEQ ID NO: 13, Nanobody 26 (Nb_B26) corresponds with SEQ ID NO: 14, Nanobody 1B3 (Nb_1B3) corresponds with SEQ ID NO: 15, Nanobody 10C2 (Nb_10C2) corresponds with SEQ ID NO: 16, Nanobody 12B6 (Nb_12B6) corresponds with SEQ ID NO: 17, Nanobody 10B5 (Nb_10B5) corresponds with SEQ ID NO: 18, Nanobody 13A5 (Nb_13A5)
35

29775-103

6

corresponds with SEQ ID NO: 19, Nanobody 2C6 (Nb2C6) corresponds with
 SEQ ID NO: 20, Nanobody 6A4 (Nb_6A4) corresponds with SEQ ID NO: 21,
 Nanobody 10C4 (Nb_10C4) corresponds with SEQ ID NO: 22, Nanobody 13B6
 (Nb_13B6) corresponds with SEQ ID NO: 23, Nanobody 1A4 (Nb_1A4) corresponds
 5 with SEQ ID NO: 24, Nanobody 2B6 (Nb_2B6) corresponds with SEQ ID NO: 25,
 Nanobody 4A2 (Nb_4A2) corresponds with SEQ ID NO: 26, Nanobody 1D4
 (Nb_1D4) corresponds with SEQ ID NO: 27 and Nanobody 9D3 (Nb_9D3)
 corresponds with SEQ ID NO: 28.

In yet another embodiment said single domain antibody is capable of inhibiting the
 10 activity of BACE1. It is understood that 'inhibition of the activity' is equivalent with the
 wording 'down-regulating the activity'. Generally, inhibition means that the activity of
 BACE1 is inhibited by at least 30%, at least 40%, at least 50%, at least 60%, at least
 70%, at least 80%, at least 90%, at least 95% or even 96%, 97%, 98%, 99% or even
 100%. Inhibition of BACE1 can be determined as mentioned herein further in the
 15 examples.

In yet another embodiment, said single domain antibody is capable of inhibiting the
 activity of BACE1 and it comprises at least one of the complementary determining
 regions (CDRs) with an amino acid sequence selected from the group comprising
 SEQ ID NOs: 29-37.

20 In yet another embodiment said single domain antibody is capable of preventing the
 uptake of pro-BACE1 and its amino acid sequence comprises SEQ ID NOs: 6, 22 or
 26.

The present invention as claimed relates to a single domain antibody, devoid of a
 light chain, specifically binding to beta-site APP-cleaving enzyme 1 (BACE1), which
 25 inhibits the activity of BACE1 as determined in a cellular assay.

It should be noted that the term Nanobody as used herein in its broadest sense is not
 limited to a specific biological source or to a specific method of preparation.

29775-103

6a

For example, the Nanobodies of the invention can generally be obtained: (1) by isolating the V_HH domain of a naturally occurring heavy chain antibody; (2) by expression of a nucleotide sequence encoding a naturally occurring V_HH domain; (3) by “humanization” of a naturally occurring V_HH domain or by expression of a nucleic acid encoding a such humanized V_HH domain; (4) by “camelization” of a naturally occurring VH domain from any animal species, and in particular from a mammalian species, such as from a human being, or by expression of a nucleic acid encoding such a camelized VH domain; (5) by “camelisation” of a “domain antibody” or “Dab” as described in the art, or by expression of a nucleic acid encoding such a camelized VH domain; (6) by using synthetic or semi-synthetic techniques for preparing proteins, polypeptides or other amino acid sequences known per se; (7) by preparing a nucleic acid encoding a Nanobody using techniques for nucleic acid synthesis known per se, followed by expression of the nucleic acid thus obtained; and/or (8) by any combination of one or more of the foregoing. One preferred class of Nanobodies corresponds to the V_HH domains of naturally occurring heavy chain antibodies directed against BACE1. As further described herein, such V_HH

sequences can generally be generated or obtained by suitably immunizing a species of Camelid with BACE1, (i.e. so as to raise an immune response and/or heavy chain antibodies directed against BACE1), by obtaining a suitable biological sample from said Camelid (such as a blood sample, serum sample or sample of B-cells), and by generating V_HH sequences directed against BACE1, starting from said sample, using any suitable technique known per se. Such techniques will be clear to the skilled person. Alternatively, such naturally occurring V_HH domains against BACE1 can be obtained from naive libraries of Camelid V_HH sequences, for example by screening such a library using BACE1 or at least one part, fragment, antigenic determinant or epitope thereof using one or more screening techniques known per se. Such libraries and techniques are for example described in WO9937681, WO0190190, WO03025020 and WO03035694. Alternatively, improved synthetic or semi-synthetic libraries derived from naive V_HH libraries may be used, such as V_HH libraries obtained from naive V_HH libraries by techniques such as random mutagenesis and/or CDR shuffling, as for example described in WO0043507. Yet another technique for obtaining V_HH sequences directed against BACE1 involves suitably immunizing a transgenic mammal that is capable of expressing heavy chain antibodies (i.e. so as to raise an immune response and/or heavy chain antibodies directed against BACE1), obtaining a suitable biological sample from said transgenic mammal (such as a blood sample, serum sample or sample of B-cells), and then generating V_HH sequences directed against BACE1 starting from said sample, using any suitable technique known per se. For example, for this purpose, the heavy chain antibody-expressing mice and the further methods and techniques described in WO02085945 and in WO04049794 can be used.

A particularly preferred class of Nanobodies of the invention comprises Nanobodies with an amino acid sequence that corresponds to the amino acid sequence of a naturally occurring V_HH domain, but that has been "humanized", i.e. by replacing one or more amino acid residues in the amino acid sequence of said naturally occurring V_HH sequence (and in particular in the framework sequences) by one or more of the amino acid residues that occur at the corresponding position(s) in a V_H domain from a conventional 4-chain antibody from a human being. This can be performed in a manner known per se, which will be clear to the skilled person, for example on the basis of the further description herein and the prior art on humanization referred to herein. Again, it should be noted that such humanized Nanobodies of the invention can be obtained in any suitable manner known per se (i.e. as indicated under points (1) - (8) above) and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring V_HH domain as a starting material.

Another particularly preferred class of Nanobodies of the invention comprises Nanobodies with an amino acid sequence that corresponds to the amino acid sequence of a naturally occurring VH domain, but that has been "camelized", i.e. by replacing one or more amino acid residues in the amino acid sequence of a naturally occurring VH domain from a conventional 4-chain antibody by one or more of the amino acid residues that occur at the corresponding position(s) in a V_HH domain of a heavy chain antibody. Such "camelizing" substitutions are preferably inserted at amino acid positions that form and/or are present at the VH-VL interface, and/or at the so-called Camelidae hallmark residues, as defined herein (see for example WO9404678). Preferably, the VH sequence that is used as a starting material or starting point for generating or designing the camelized Nanobody is preferably a VH sequence from a mammal, more preferably the VH sequence of a human being, such as a VH3 sequence. However, it should be noted that such camelized Nanobodies of the invention can be obtained in any suitable manner known per se (i.e. as indicated under points (1) - (8) above) and thus are not strictly limited to polypeptides that have been obtained using a polypeptide that comprises a naturally occurring VH domain as a starting material. For example both "humanization" and "camelization" can be performed by providing a nucleotide sequence that encodes a naturally occurring V_HH domain or VH domain, respectively, and then changing, in a manner known per se, one or more codons in said nucleotide sequence in such a way that the new nucleotide sequence encodes a "humanized" or "camelized" Nanobody of the invention, respectively. This nucleic acid can then be expressed in a manner known per se, so as to provide the desired Nanobody of the invention. Alternatively, based on the amino acid sequence of a naturally occurring V_HH domain or VH domain, respectively, the amino acid sequence of the desired humanized or camelized Nanobody of the invention, respectively, can be designed and then synthesized de novo using techniques for peptide synthesis known per se. Also, based on the amino acid sequence or nucleotide sequence of a naturally occurring V_HH domain or VH domain, respectively, a nucleotide sequence encoding the desired humanized or camelized Nanobody of the invention, respectively, can be designed and then synthesized de novo using techniques for nucleic acid synthesis known per se, after which the nucleic acid thus obtained can be expressed in a manner known per se, so as to provide the desired Nanobody of the invention. Other suitable methods and techniques for obtaining the Nanobodies of the invention and/or nucleic acids encoding the same, starting from naturally occurring VH sequences or preferably V_HH sequences, will be clear from the skilled person, and may for example comprise combining one or more parts of one or more naturally occurring VH sequences (such as one or more FR sequences and/or CDR sequences), one or more parts of one or more naturally occurring V_HH sequences (such as one or more FR sequences or CDR

sequences), and/or one or more synthetic or semi-synthetic sequences, in a suitable manner, so as to provide a Nanobody of the invention or a nucleotide sequence or nucleic acid encoding the same.

According to one non-limiting aspect of the invention, a Nanobody may be as defined herein,

5 but with the proviso that it has at least "one amino acid difference" (as defined herein) in at least one of the framework regions compared to the corresponding framework region of a naturally occurring human VH domain, and in particular compared to the corresponding framework region of DP-47. More specifically, according to one non-limiting aspect of the invention, a Nanobody may be as defined herein, but with the proviso that it has at least "one

10 amino acid difference" (as defined herein) at at least one of the Hallmark residues (including those at positions 108, 103 and/or 45) compared to the corresponding framework region of a naturally occurring human VH domain, and in particular compared to the corresponding framework region of DP-47. Usually, a Nanobody will have at least one such amino acid difference with a naturally occurring VH domain in at least one of FR2 and/or FR4, and in

15 particular at at least one of the Hallmark residues in FR2 and/or FR4 (again, including those at positions 108, 103 and/or 45). Also, a humanized Nanobody of the invention may be as defined herein, but with the proviso that it has at least "one amino acid difference" (as defined herein) in at least one of the framework regions compared to the corresponding framework region of a naturally occurring V_HH domain. More specifically, according to one non-limiting

20 aspect of the invention, a Nanobody may be as defined herein, but with the proviso that it has at least "one amino acid difference" (as defined herein) at at least one of the Hallmark residues (including those at positions 108, 103 and/or 45) compared to the corresponding framework region of a naturally occurring V_HH domain. Usually, a Nanobody will have at least one such amino acid difference with a naturally occurring V_HH domain in at least one of FR2 and/or FR4,

25 and in particular at at least one of the Hallmark residues in FR2 and/or FR4 (again, including those at positions 108, 103 and/or 45). As will be clear from the disclosure herein, it is also within the scope of the invention to use natural or synthetic analogs, mutants, variants, alleles, homologs and orthologs (herein collectively referred to as "analogs") of the Nanobodies of the invention as defined herein, and in particular analogs of the Nanobodies of SEQ ID NOs: 6, 22

30 or 26. Thus, according to one embodiment of the invention, the term "Nanobody of the invention" in its broadest sense also covers such analogs. Generally, in such analogs, one or more amino acid residues may have been replaced, deleted and/or added, compared to the Nanobodies of the invention as defined herein. Such substitutions, insertions or deletions may be made in one or more of the framework regions and/or in one or more of the CDR's, and in

particular analogs of the CDR's of the Nanobodies of SEQ ID NOs: 6, 22 or 26, said CDR's corresponding with SEQ ID NOs: 29-37 (see Table 1, Fig. 1, Fig. 5).

Table 1: CDRs of BACE1-specific nanobodies

Nb	CDR1	CDR2	CDR3
Nb_B9 (SEQ ID NO: 6)	EYTYGYCSMG (SEQ ID NO 29)	TITSDGSTSYVDSVKG (SEQ ID NO 30)	KTCANKLGAKFIS (SEQ ID NO 31)
Nb_10C4 (SEQ ID NO: 22)	GYTYSTCSMA (SEQ ID NO 32)	SIRNDGSTAYADSVKG (SEQ ID NO 33)	RIGVGPGGTCSIYAPY (SEQ ID NO 34)
Nb_4A2 (SEQ ID NO: 26)	GFTFETQYMT (SEQ ID NO 35)	SINSGGTIKYYANSSVKG (SEQ ID NO 36)	GQWAGVGAASS (SEQ ID NO 37)

5

When such substitutions, insertions or deletions are made in one or more of the framework regions, they may be made at one or more of the Hallmark residues and/or at one or more of the other positions in the framework residues. Substitutions, insertions or deletions at the Hallmark residues are generally less preferred (unless these are suitable humanizing substitutions as described herein). By means of non-limiting examples, a substitution may for example be a conservative substitution (as described herein) and/or an amino acid residue may be replaced by another amino acid residue that naturally occurs at the same position in another V_HH domain. Thus, any one or more substitutions, deletions or insertions, or any combination thereof, that either improve the properties of the Nanobody of the invention or that at least do not detract too much from the desired properties or from the balance or combination of desired properties of the Nanobody of the invention (i.e. to the extent that the Nanobody is no longer suited for its intended use) are included within the scope of the invention. A skilled person will generally be able to determine and select suitable substitutions, deletions or insertions, or suitable combinations of thereof, based on the disclosure herein and optionally after a limited degree of routine experimentation, which may for example involve introducing a limited number of possible substitutions and determining their influence on the properties of the Nanobodies thus obtained. For example, and depending on the host organism used to express the Nanobody or polypeptide of the invention, such deletions and/or substitutions may be designed in such a way that one or more sites for post-translational modification (such as one or more glycosylation sites) are removed, as will be within the ability of the person skilled in the

25

art. Alternatively, substitutions or insertions may be designed so as to introduce one or more sites for attachment of functional groups (as described herein), for example to allow site-specific pegylation. One preferred class of analogs of the Nanobodies of the invention comprise Nanobodies that have been humanized (i.e. compared to the sequence of a naturally occurring Nanobody of the invention). As mentioned in the background art cited herein, such humanization generally involves replacing one or more amino acid residues in the sequence of a naturally occurring V_HH with the amino acid residues that occur at the same position in a human VH domain, such as a human VH3 domain. Examples of possible humanizing substitutions or combinations of humanizing substitutions will be clear to the skilled person, from the possible humanizing substitutions mentioned in the background art cited herein, and/or from a comparison between the sequence of a Nanobody and the sequence of a naturally occurring human VH domain. The humanizing substitutions should be chosen such that the resulting humanized Nanobodies still retain the favourable properties of Nanobodies as defined herein, and more preferably such that they are as described for analogs in the preceding paragraphs. A skilled person will generally be able to determine and select suitable humanizing substitutions or suitable combinations of humanizing substitutions, based on the disclosure herein and optionally after a limited degree of routine experimentation, which may for example involve introducing a limited number of possible humanizing substitutions and determining their influence on the properties of the Nanobodies thus obtained. Generally, as a result of humanization, the Nanobodies of the invention may become more "human-like", while still retaining the favorable properties of the Nanobodies of the invention as described herein. As a result, such humanized Nanobodies may have several advantages, such as a reduced immunogenicity, compared to the corresponding naturally occurring V_HH domains. Again, based on the disclosure herein and optionally after a limited degree of routine experimentation, the skilled person will be able to select humanizing substitutions or suitable combinations of humanizing substitutions which optimize or achieve a desired or suitable balance between the favourable properties provided by the humanizing substitutions on the one hand and the favourable properties of naturally occurring V_HH domains on the other hand. Examples of such modifications, as well as examples of amino acid residues within the Nanobody sequence that can be modified in such a manner (i.e. either on the protein backbone but preferably on a side chain), methods and techniques that can be used to introduce such modifications and the potential uses and advantages of such modifications will be clear to the skilled person. For example, such a modification may involve the introduction (e.g. by covalent linking or in another suitable manner) of one or more functional groups, residues or moieties into or onto the Nanobody of the invention, and in particular of one or more functional groups, residues or

moieties that confer one or more desired properties or functionalities to the Nanobody of the invention. Example of such functional groups will be clear to the skilled person. Examples of such functional groups and of techniques for introducing them will be clear to the skilled person, and can generally comprise all functional groups and techniques mentioned in the general background art cited hereinabove as well as the functional groups and techniques known per se for the modification of pharmaceutical proteins, and in particular for the modification of antibodies or antibody fragments (including ScFv's and single domain antibodies), for which reference is for example made to Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, PA (1980). Such functional groups may for example be linked directly (for example covalently) to a Nanobody of the invention, or optionally via a suitable linker or spacer, as will again be clear to the skilled person. One of the most widely used techniques for increasing the half-life and/or reducing immunogenicity of pharmaceutical proteins comprises attachment of a suitable pharmacologically acceptable polymer, such as poly(ethyleneglycol) (PEG) or derivatives thereof (such as methoxypoly(ethyleneglycol) or mPEG). Generally, any suitable form of pegylation can be used, such as the pegylation used in the art for antibodies and antibody fragments (including but not limited to (single) domain antibodies and ScFv's); reference is made to for example Chapman, Nat. Biotechnol., 54, 531-545 (2002); by Veronese and Harris, Adv. Drug Deliv. Rev. 54, 453-456 (2003), by Harris and Chess, Nat. Rev. Drug. Discov., 2, (2003) and in WO04060965. Various reagents for pegylation of proteins are also commercially available, for example from Nektar Therapeutics, USA. Preferably, site-directed pegylation is used, in particular via a cysteine-residue (see for example Yang et al., Protein Engineering, 16, 10, 761-770 (2003). For example, for this purpose, PEG may be attached to a cysteine residue that naturally occurs in a Nanobody of the invention, a Nanobody of the invention may be modified so as to suitably introduce one or more cysteine residues for attachment of PEG, or an amino acid sequence comprising one or more cysteine residues for attachment of PEG may be fused to the N- and/or C-terminus of a Nanobody of the invention, all using techniques of protein engineering known per se to the skilled person. Preferably, for the Nanobodies and proteins of the invention, a PEG is used with a molecular weight of more than 5000, such as more than 10,000 and less than 200,000, such as less than 100,000; for example in the range of 20,000-80,000. Another, usually less preferred modification comprises N-linked or O-linked glycosylation, usually as part of co-translational and/or post-translational modification, depending on the host cell used for expressing the Nanobody or polypeptide of the invention. Yet another modification may comprise the introduction of one or more detectable labels or other signal-generating groups or moieties, depending on the intended use of the labelled Nanobody. Suitable labels and

techniques for attaching, using and detecting them will be clear to the skilled person, and for example include, but are not limited to, fluorescent labels (such as fluorescein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthalaldehyde, and fluorescamine and fluorescent metals such as Eu or others metals from the lanthanide series),
5 phosphorescent labels, chemiluminescent labels or bioluminescent labels (such as luminal, isoluminol, thermotropic acridinium ester, imidazole, acridinium salts, oxalate ester, dioxetane or GFP and its analogs), radio-isotopes, metals, metals chelates or metallic cations or other metals or metallic cations that are particularly suited for use in in vivo, in vitro or in situ diagnosis and imaging, as well as chromophores and enzymes (such as malate
10 dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, biotinavidin peroxidase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholine esterase). Other suitable labels will be clear
15 to the skilled person, and for example include moieties that can be detected using NMR or ESR spectroscopy. Such labelled Nanobodies and polypeptides of the invention may for example be used for in vitro, in vivo or in situ assays (including immunoassays known per se such as ELISA, RIA, EIA and other "sandwich assays", etc.) as well as in vivo diagnostic and imaging purposes, depending on the choice of the specific label. As will be clear to the skilled
20 person, another modification may involve the introduction of a chelating group, for example to chelate one of the metals or metallic cations referred to above. Suitable chelating groups for example include, without limitation, diethyl- enetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA). Yet another modification may comprise the introduction of a functional group that is one part of a specific binding pair, such as the biotin-
25 (strept)avidin binding pair. Such a functional group may be used to link the Nanobody of the invention to another protein, polypeptide or chemical compound that is bound to the other half of the binding pair, i.e. through formation of the binding pair. For example, a Nanobody of the invention may be conjugated to biotin, and linked to another protein, polypeptide, compound or carrier conjugated to avidin or streptavidin. For example, such a conjugated Nanobody may be
30 used as a reporter, for example in a diagnostic system where a detectable signal-producing agent is conjugated to avidin or streptavidin. Such binding pairs may for example also be used to bind the Nanobody of the invention to a carrier, including carriers suitable for pharmaceutical purposes. One non-limiting example are the liposomal formulations described by Cao and Suresh, Journal of Drug Targeting, 8, 4, 257 (2000). Such binding pairs may also be used to
35 link a therapeutically active agent to the Nanobody of the invention.

It is expected that the Nanobodies and polypeptides of the invention will generally bind to all naturally occurring or synthetic analogs, variants, mutants, alleles, parts and fragments of BACE1, or at least to those analogs, variants, mutants, alleles, parts and fragments of BACE1, that contain one or more antigenic determinants or epitopes that are essentially the same as the antigenic determinant(s) or epitope(s) to which the Nanobodies and polypeptides of the invention bind in BACE1 (e.g. in wild-type BACE1,). Again, in such a case, the Nanobodies and polypeptides of the invention may bind to such analogs, variants, mutants, alleles, parts and fragments with an affinity and/or specificity that are the same as, or different from (i.e. higher than or lower than), the affinity and specificity with which the Nanobodies of the invention bind to (wild-type) BACE1. It is also included within the scope of the invention that the Nanobodies and polypeptides of the invention bind to some analogs, variants, mutants, alleles, parts and fragments of BACE1, but not to others. Also, in determining the degree of sequence identity between two amino acid sequences, the skilled person may take into account so-called "conservative" amino acid substitutions, which can generally be described as amino acid substitutions in which an amino acid residue is replaced with another amino acid residue of similar chemical structure and which has little or essentially no influence on the function, activity or other biological properties of the polypeptide. Such conservative amino acid substitutions are well known in the art, for example from WO04037999, WO9849185, WO0046383 and WO0109300; and (preferred) types and/or combinations of such substitutions may be selected on the basis of the pertinent teachings from WO04037999 as well as WO9849185. Such conservative substitutions preferably are substitutions in which one amino acid within the following groups (a) - (e) is substituted by another amino acid residue within the same group: (a) small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly; (b) polar, negatively charged residues and their (uncharged) amides: Asp, Asn, Glu and Gln; (c) polar, positively charged residues: His, Arg and Lys; (d) large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and (e) aromatic residues: Phe, Tyr and Trp. Particularly preferred conservative substitutions are as follows: Ala into Gly or into Ser; Arg into Lys; Asn into Gln or into His; Asp into Glu; Cys into Ser; Gln into Asn; Glu into Asp; Gly into Ala or into Pro; His into Asn or into Gln; Ile into Leu or into Val; Leu into Ile or into Val; Lys into Arg, into Gln or into Glu; Met into Leu, into Tyr or into Ile; Phe into Met, into Leu or into Tyr; Ser into Thr; Thr into Ser; Trp into Tyr; Tyr into Trp; and/or Phe into Val, into Ile or into Leu.

Polypeptide therapeutics and in particular antibody-based therapeutics have significant potential as drugs because they have exquisite specificity to their target and a low inherent toxicity. However, it is known by the skilled person that an antibody which has been obtained

for a therapeutically useful target requires additional modification in order to prepare it for human therapy, so as to avoid an unwanted immunological reaction in a human individual upon administration. The modification process is commonly termed "humanization". It is known by the skilled artisan that antibodies raised in species, other than in humans, require humanization to render the antibody therapeutically useful in humans ((1) CDR grafting : Protein Design Labs: US6180370, US5693761; Genentech US6054297; Celltech: EP626390, US5859205; (2) Veneering: Xoma: US5869619, US5766886, US5821123). There is a need for a method for producing antibodies which avoids the requirement for substantial humanization, or which completely obviates the need for humanization. There is a need for a new class of antibodies which have defined framework regions or amino acid residues and which can be administered to a human subject without the requirement for substantial humanization, or the need for humanization at all. According to one aspect of the invention, Nanobodies are polypeptides which are derived from heavy chain antibodies and whose framework regions and complementary determining regions are part of a single domain polypeptide. Examples of such heavy chain antibodies include, but are not limited to, naturally occurring immunoglobulins devoid of light chains. Such immunoglobulins are disclosed in WO9404678 for example. The antigen-binding site of this unusual class of heavy chain antibodies has a unique structure that comprises a single variable domain. For clarity reasons, the variable domain derived from a heavy chain antibody naturally devoid of light chain is known herein as a V_{HH} or V_HH domain or nanobody. Such a V_{HH} domain peptide can be derived from antibodies raised in Camelidae species, for example in camel, dromedary, llama, alpaca and guanaco. Other species besides Camelidae (e.g. shark, pufferfish) may produce functional antigen-binding heavy chain antibodies naturally devoid of light chain. V_{HH} domains derived from such heavy chain antibodies are within the scope of the invention. Camelidae antibodies express a unique, extensive repertoire of functional heavy chain antibodies that lack light chains. The V_{HH} molecules derived from Camelidae antibodies are the smallest intact antigen-binding domains known (approximately 15 kDa, or 10 times smaller than conventional IgG) and hence are well suited towards delivery to dense tissues and for accessing the limited space between macromolecules. Other examples of Nanobodies include Nanobodies derived from V_H domains of conventional four chain antibodies which have been modified by substituting one or more amino acid residues with Camelidae-specific residues (the so-called camelisation of heavy chain antibodies, WO9404678). Such positions may preferentially occur at the V_H - V_L interface and at the so-called Camelidae hallmark residues (WO9404678), comprising positions 37, 44, 45, 47, 103 and 108. Nanobodies correspond to small, robust and efficient recognition units formed by a single immunoglobulin (Ig) domain.

A fragment of a nanobody as used herein refers to less than 100% of the sequence (e.g., 99%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% etc.), but comprising 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more amino acids. A fragment is preferably of sufficient length such that the interaction of interest is maintained with affinity of 1×10^{-6} M or better. A fragment as used herein also refers to optional insertions, deletions and substitutions of one or more amino acids which do not substantially alter the ability of the target to bind to a Nanobody raised against the wild-type target. The number of amino acid insertions deletions or substitutions is preferably up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41; 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70 amino acids. One embodiment of the present invention relates to a polypeptide comprising at least one nanobody wherein one or more amino acid residues have been substituted without substantially altering the antigen binding capacity.

In a particular embodiment, the antibody of the invention is bivalent and formed by bonding, chemically or by recombinant DNA techniques, together two monovalent single domain of heavy chains. In another particular embodiment the antibody of the invention is bi-specific and formed by bonding together two variable domains of heavy chains, each with a different specificity (id est one with a specificity for BACE1 and the other one with a specificity for a neuron such as for example ICAM5 or telencephalin). Similarly, polypeptides comprising multivalent or multi-specific single domain antibodies are included here as non-limiting examples.

In yet another embodiment a single domain antibody which is capable of preventing the uptake of BACE1 can be used as a medicament. In yet another embodiment, a single domain antibody that comprises at least one of the complementary determining regions (CDRs) with an amino acid sequence selected from the group comprising SEQ ID NOs: 29-37 can be used as a medicament. In yet another embodiment a single domain antibody which amino acid comprises SEQ ID NOs: 6, 22 or 26 can be used as a medicament.

In yet another embodiment a single domain antibody which is capable of preventing the uptake of pro-BACE1 can be used for the manufacture of a medicament to treat diseases associated with an overexpression of BACE1. An examples of a disease where an overexpression of BACE1 occurs is Alzheimer's disease. In general, "therapeutically effective amount", "therapeutically effective dose" and "effective amount" means the amount needed to achieve

the desired result or results (inhibiting BACE1 binding; treating or preventing Alzheimer's disease). One of ordinary skill in the art will recognize that the potency and, therefore, an "effective amount" can vary for the nanobody that inhibits BACE1 binding used in the invention. One skilled in the art can readily assess the potency of the nanobody. By

5 "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the compound without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

The term 'medicament to treat' relates to a composition comprising antibodies as described

10 above and a pharmaceutically acceptable carrier or excipient (both terms can be used interchangeably) to treat or to prevent diseases as described herein. The administration of a nanobody as described above or a pharmaceutically acceptable salt thereof may be by way of oral, inhaled or parenteral administration. In particular embodiments the nanobody is delivered through intrathecal or intracerebroventricular administration. The active compound may be

15 administered alone or preferably formulated as a pharmaceutical composition. An amount effective to treat Alzheimer's disease that express the antigen recognized by the nanobody depends on the usual factors such as the nature and severity of the disorder being treated and the weight of the mammal. However, a unit dose will normally be in the range of 0.01 to 50 mg, for example 0.01 to 10 mg, or 0.05 to 2 mg of nanobody or a pharmaceutically acceptable salt

20 thereof. Unit doses will normally be administered once or more than once a day, for example 2, 3, or 4 times a day, more usually 1 to 3 times a day, such that the total daily dose is normally in the range of 0.0001 to 1 mg/kg; thus a suitable total daily dose for a 70 kg adult is 0.01 to 50 mg, for example 0.01 to 10 mg or more usually 0.05 to 10 mg. It is greatly preferred that the compound or a pharmaceutically acceptable salt thereof is administered in the form of a unit-

25 dose composition, such as a unit dose oral, parenteral, or inhaled composition. Such compositions are prepared by admixture and are suitably adapted for oral, inhaled or parenteral administration, and as such may be in the form of tablets, capsules, oral liquid preparations, powders, granules, lozenges, reconstitutable powders, injectable and infusable solutions or suspensions or suppositories or aerosols. Tablets and capsules for oral

30 administration are usually presented in a unit dose, and contain conventional excipients such as binding agents, fillers, diluents, tableting agents, lubricants, disintegrants, colourants, flavourings, and wetting agents. The tablets may be coated according to well known methods in the art. Suitable fillers for use include cellulose, mannitol, lactose and other similar agents. Suitable disintegrants include starch, polyvinylpyrrolidone and starch derivatives such as

35 sodium starch glycollate. Suitable lubricants include, for example, magnesium stearate.

Suitable pharmaceutically acceptable wetting agents include sodium lauryl sulphate. These solid oral compositions may be prepared by conventional methods of blending, filling, tableting or the like. Repeated blending operations may be used to distribute the active agent throughout those compositions employing large quantities of fillers. Such operations are, of course, conventional in the art. Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups, or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, for example sorbitol, syrup, methyl cellulose, gelatin, hydroxyethylcellulose, carboxymethyl cellulose, aluminium stearate gel or hydrogenated edible fats, emulsifying agents, for example lecithin, sorbitan monooleate, or acacia; non-aqueous vehicles (which may include edible oils), for example, almond oil, fractionated coconut oil, oily esters such as esters of glycerine, propylene glycol, or ethyl alcohol; preservatives, for example methyl or propyl p-hydroxybenzoate or sorbic acid, and if desired conventional flavouring or colouring agents. Oral formulations also include conventional sustained release formulations, such as tablets or granules having an enteric coating. Preferably, compositions for inhalation are presented for administration to the respiratory tract as a snuff or an aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose. In such a case the particles of active compound suitably have diameters of less than 50 microns, preferably less than 10 microns, for example between 1 and 5 microns, such as between 2 and 5 microns. A favored inhaled dose will be in the range of 0.05 to 2 mg, for example 0.05 to 0.5 mg, 0.1 to 1 mg or 0.5 to 2 mg. For parenteral administration, fluid unit dose forms are prepared containing a compound of the present invention and a sterile vehicle. The active compound, depending on the vehicle and the concentration, can be either suspended or dissolved. Parenteral solutions are normally prepared by dissolving the compound in a vehicle and filter sterilising before filling into a suitable vial or ampoule and sealing. Advantageously, adjuvants such as a local anaesthetic, preservatives and buffering agents are also dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under vacuum. Parenteral suspensions are prepared in substantially the same manner except that the compound is suspended in the vehicle instead of being dissolved and sterilised by exposure to ethylene oxide before suspending in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in the composition to facilitate uniform distribution of the active compound. Where appropriate, small amounts of bronchodilators for example sympathomimetic amines such as isoprenaline, isoetharine, salbutamol, phenylephrine and ephedrine; xanthine derivatives such as theophylline and

aminophylline and corticosteroids such as prednisolone and adrenal stimulants such as ACTH may be included. As is common practice, the compositions will usually be accompanied by written or printed directions for use in the medical treatment concerned.

- 5 In yet another embodiment of the invention, one or more single domain antibodies of the invention can be linked (optionally via one or more suitable linker sequences) to one or more (such as two and preferably one) amino acid sequences that allow the resulting polypeptide of the invention to cross the blood brain barrier. In particular, said one or more amino acid sequences that allow the resulting polypeptides of the invention to cross the blood brain barrier
- 10 may be one or more (such as two and preferably one) nanobodies, such as the nanobodies described in WO 02/057445, of which FC44 (SEQ ID NO: 189 of WO 06/040153) and FC5 (SEQ ID NO: 190 of WO 06/040154) are preferred examples.

The present invention further provides a pharmaceutical composition for use in the treatment and/or prophylaxis of herein described disorders which comprises a pharmaceutically acceptable salt thereof, or a pharmaceutically acceptable solvate thereof, and, if required, a pharmaceutically acceptable carrier thereof.

It should be clear that the therapeutic method of the present invention for Alzheimer's disease can also be used in combination with any other AD disease therapy known in the art such as gamma-secretase inhibitors, other beta-secretase inhibitors.

In a particular embodiment the single domain antibodies of the present invention can be used for the preparation of a diagnostic assay. BACE1 can be detected in a variety of cells and tissues, especially in brain cells and tissues, wherein the degree of expression corroborates with the severity of Alzheimer's disease. Therefore, there is provided a method of *in situ* detecting localization and distribution of BACE1 expression in a biological sample. The method comprises the step of reacting the biological sample with a detectable BACE1 nanobody and detecting the localization and distribution of the detectable nanobody. The term "biological sample" refers to cells and tissues, including, but not limited to brain cells and tissues. The term further relates to body fluids. Therefore, there is provided a method of detecting BACE1 protein in a body fluid of a patient. The method comprises the steps of reacting the body fluid with an anti-BACE1 nanobody of the present invention and monitoring the reaction. The body fluid is, for example, plasma, urine, cerebrospinal fluid, pleural effusions or saliva. Monitoring the reaction may be effected by having the nanobody labeled with a detectable moiety, or to use its constant region as an inherent detectable moiety, to which a second antibody which includes a detectable moiety can specifically bind. CSF BACE1 can for example be detected in

patients suffering from Alzheimer's disease. According to a preferred embodiment of the present invention reacting the body fluid with the anti-BACE1 nanobody is effected in solution. Alternatively, reacting the body fluid with the anti-BACE1 nanobody is effected on a substrate capable of adsorbing proteins present in the body fluid, all as well known in the art of antibody based diagnosis. Further according to the present invention there is provided a method of detecting the presence, absence or level of BACE1 protein in a biological sample. The method comprises the following steps. First, proteins are extracted from the biological sample, thereby a plurality of proteins are obtained. The protein extract may be a crude extract and can also include non-proteinaceous material. Second, the proteins are size separated, e.g., by electrophoresis, gel filtration etc. Fourth, the size separated proteins are interacted with an anti-BACE1 nanobody. Finally, the presence, absence or level of the interacted anti-BACE1 nanobody is detected. In case of gel electrophoresis the interaction with the nanobody is typically performed following blotting of the size separated proteins onto a solid support (membrane).

The following examples more fully illustrate preferred features of the invention, but are not intended to limit the invention in any way. All of the starting materials and reagents disclosed below are known to those skilled in the art, and are available commercially or can be prepared using well-known techniques.

Examples

1. Generation and isolation of BACE1-specific nanobodies

To generate BACE1-specific antibodies a dromedary was immunized six times with recombinant human BACE1 over a period of about 6 weeks. After this period a BACE1-specific humoral response, as assessed by ELISA, was observed for each of the 3 different IgG subclasses that exist in Camelidae, namely the conventional IgG1 molecules and the heavy chain-only subclasses IgG2 and IgG3 (IgG classes reviewed in Conrath et al., 2003). The variable chain of the HCAs (V_{HH}), which contain the antigen-binding fragment, was amplified from isolated dromedary lymphocytes and cloned into a pHEN4 phagemid vector to generate a library of 4×10^7 individual transformants. After rescuing this bank with M13K07 helper phages, the V_{HH} repertoire was expressed on the surface of bacteriophages. With these phages BACE1-specific V_{HH} s could be isolated from the whole V_{HH} pool using panning, an *in vitro* selection technique (reviewed in Smith and Petrenko, 1997). For this, the phages were incubated onto a solid phase passively coated with the immunogen. After washing, bound phages were eluted and used to infect exponentially growing *E. coli* TG1 cells to produce new

virions. These virions were used in a next selection round in order to enrich for BACE1-specific binders. After 2 to 3 consecutive rounds of panning, individual colonies were randomly picked and incubated with IPTG to induce expression of the nanobodies. The V_HH protein fragments were extracted from the bacterial periplasm and tested individually by ELISA for their ability to interact with BACE1. The positive scoring clones were sequenced and as such 20 different specific nanobodies were identified.

2. Sequence analysis of the BACE1-binders

Fourteen out of the twenty selected BACE1-binders are clearly derived from V_HH germ-line genes: Nb_B1, Nb_B2, Nb_B3, Nb_B5, Nb_B8, Nb_B9, Nb_B10, Nb_B11, Nb_B12, Nb_B15, Nb_B16, Nb_B21, Nb_B25, and Nb_B26, corresponding with SEQ ID NOs: 1-14, respectively (Fig. 1). The amino acid sequence of their framework-2 (FR2) region resembles that of a typical V_HH FR2 (Muyldermans et al., 1994), with residues F/Y, E/Q, R/C and G at positions 37, 44, 45 and 47 respectively (numbering according to Kabat et al., 1991). However, the remaining six antibody fragments, Nb_B4, Nb_B6, Nb_B7, Nb_B13, Nb_B14 and Nb_B24 (corresponding with SEQ ID NOs: 38-43, respectively), seem to originate from conventional antibody germ-line genes, since they contain the V₃₇G₄₄L₄₅W₄₇ tetrad, a typical hallmark that distinguishes the variable domain of the heavy chain of conventional antibodies (V_H) from V_HH fragments at the germline level. These hallmark residues are critically required in H₂-L₂ antibodies for the association of the heavy chain with a light chain. Due to the high sequence similarity of the 6 V_H-like nanobodies, it is most likely they are all derived from one and the same B cell lineage. The differences in amino acid sequence could be the result of the ongoing somatic hypermutation of the antibody gene fragments in maturing B cells and the subsequent antigen-driven selection, a continuous process leading to ever better fitting antibodies. The 6 V_H-like nanobodies also differ from the other binders in that they contain a leucine residue at position 11 in their framework-1 (FR1), another characteristic of V_H-genes that is important for the interaction with a light chain (Lesk and Chothia, 1988, Padlan, 1994). In a typical V_HH FR1 this Leu residue is often replaced by a smaller and hydrophilic residue, usually a serine, as seen in the 14 BACE1-binders with a true V_HH motif. The V_H-like molecules all have a short CDR3 of only 6 amino acids, whereas the other binders have significantly larger H3 loops, ranging from 13 to 21 residues, with an average length of 17. This is consistent with the average V_HH-CDR3 length of 15-16 residues reported before in literature (reviewed in Muyldermans and Lauwereys, 1999). In general the CDR2 and CDR1 of V_HHs consist of 16-17 and 10 residues respectively, but about 30% of dromedary V_HH cDNAs were reported to be off-sized. This does not adversely affect their function, but instead even increases the antigen

binding repertoire (Nguyen et al., 2000). Unusual CDR1 and CDR2 lengths are also observed for our BACE1 binders. The CDR2 of Nb_B15 contains 19 residues due to a tandem repetition of 2 amino acids, whereas that of Nb_B21 consists of 18 residues. Aberrant CDR1 sizes are found in Nb_B1, Nb_B15 and Nb_B16 due to an insertion, a deletion of 1 amino acid and a deletion of 2 residues, respectively. Finally, Nb_B25 has an unusually long framework-3 region with a tandem repetition of 2 amino acid residues. Deviating lengths in the BACE1-binders are due to changes located at 3 typical V_HH insertion / deletion hot spots, surrounding residues 30 ± 3 , 54 ± 3 and 74 ± 1 . These hot spots can be found within or at the border of peculiar DNA sequences, such as palindromic sequences (corresponding to residues 30-33 and 54-57) or heptamer-like sequences of an Ig recombination signal (often found at residues 76-78) (Nguyen et al., 2000). Besides the conserved disulphide bridge between Cys₂₂ and Cys₉₂, extra non-canonical cysteine residues do not frequently occur in conventional antibodies, although they are not totally excluded. However, an additional pair of cysteines is encountered in 75 % of reported dromedary V_HHs (Arbabi Ghahroudi et al., 1997, Lauwereys et al., 1998, Conrath et al., 2001a, Saerens et al., 2004). One of these extra cysteine residues is typically located within the CDR3 loop, whereas the other one can be found either on position 30, 32 or 33 within the CDR1 or at position 45 in FR2. Since the V_HH CDR3 loop folds back onto the CDR1-FR2 region, the 2 cysteine residues come into contact distance and are likely engaged into an interloop disulphide bond that crosslinks the antigen-binding loops (Desmyter et al., 1996). Such a bond reduces flexibility of the long CDR3 loop and thus provides increased stability. Besides, the interloop bond might lead to a constrained, but new conformation of the CDR loops, thereby increasing the antigen-binding repertoire.

Compared to the percentages known from literature, there is a low incidence of additional cysteines in the BACE1 binders. A putative additional disulphide bond is only present in 4 out of the 14 nanobodies with V_HH motif. Nb_B25 has a cysteine residue at position 33 within the CDR1, Nb_B9 has one at position 32 and in Nb_B5 an additional bridge will probably be formed between Cys₄₅ and the CDR3. A cysteine at position 53, as seen in Nb_B12, has been described so far for neither dromedary, nor llama V_HHs.

3. Defining affinities of the BACE1-binders for their immunogen at pH7.0 and pH5.0

The cDNAs of the 20 isolated BACE1-binders were subcloned into the pHEN6 prokaryotic expression vector and expressed in *E. coli* WK6 cells to produce his-tagged soluble proteins. The recombinant V_HHs were subsequently purified by Ni-NTA affinity chromatography, followed by size-exclusion chromatography. The expression levels of the distinct clones varied between 1 and 15 mg per litre of culture medium. The affinity of all V_HHs for BACE1 was

determined quantitatively using the surface plasmon resonance technology on Biacore 3000. Each of the different V_HHs was injected at concentrations ranging from 0 to 0.5 μ M on a chip onto which BACE1 was coupled. Binding was evaluated both at pH7.0 and pH5.0. Measurements at pH5.0 were included because a firm interaction between BACE1 and a V_HH should be preserved at this pH, since the endosomal compartment with its slightly acidic content was reported to be the major subcellular site of β -site cleavage of APP (Koo, 1994). Besides, BACE1 was shown to have optimal β -secretase activity at about pH5.0 *in vitro* (Sinha et al., 1999, Vassar et al., 1999, Yan et al., 1999, Lin et al., 2000). The dissociation constants obtained for all V_HHs vary between 4 and 669 nM at pH7.0 and between 4.2 nM and 6.8 μ M at pH5.0 (**Fig. 2A**). The majority of the binders have dissociation constants between 10 and 100 nM at both pH conditions.

4. Capacity of the different V_HHs to pull down native BACE1

For the immunization of the dromedary, the isolation of the BACE1-specific binders during panning and the *in vitro* affinity measurements by Biacore, we used recombinant human soluble BACE1, completely devoid of carbohydrate chains. This recombinant protein, supplied by Dr. S. Masure (Johnson & Johnson, Beerse, Belgium) was obtained from an insect cell expression system using slightly truncated BACE1 cDNA in which the 4 N-glycosylation sites and the whole membrane anchor were removed (Bruinzeel et al., 2002). It is not unthinkable that epitopes that are easily accessible in the “naked” BACE1, used for the immunization, are shielded by glycan chains or other posttranslational modifications of BACE1 proteins generated in mammalian cells. Therefore, we wondered whether the selected binders would all be able to recognize glycosylated BACE1 expressed in mammalian cells.

To test this, 2 μ g of his-tagged V_HH molecules were incubated with 4 μ g of total protein extract from COS cells transiently transfected with human BACE1 cDNA. Nickel-beads were subsequently used to pull down the V_HH molecules together with the bound proteins. After extensive washing bound proteins were eluted, separated by SDS-PAGE and BACE1 protein was detected by Western blotting using a rabbit polyclonal BACE1 specific antibody (ProSci, 2253) (**Fig. 2B**). V_HH proteins raised against either A β (Nb_A β 3) or beta-lactamase BCII 569/H (Nb_BCIIIP) (Conrath et al., 2001a), were used as negative controls and were unable to capture BACE1 from the cell lysate, as expected. Five binders, Nb_B7, Nb_B9, Nb_B10, Nb_B13 and Nb_B24 have the highest efficacy to pull down BACE1 compared to the other nanobodies. For Nb_B3, Nb_B8, Nb_B12 and Nb_B21 at the best a trifling trace of coprecipitated BACE1 can be detected after overnight exposure.

Note that in the group of the 6 V_H-like nanobodies (Nb_B4, Nb_B6, Nb_B7, Nb_B13, Nb_B14 and Nb_B24) huge differences are observed in the ability of each nanobody to bind to glycosylated BACE1, even though they probably originated from the same B-cell lineage. Despite a high overall sequence similarity of about 90%, Nb_B13 and Nb_B14 share only 70% of amino acids in their antigen-binding CDR regions and this difference apparently is sufficient to adversely affect the affinity of Nb_B14 for its antigen when compared to Nb_B13.

5. Effect of ectopic expression of the nanobodies on BACE inhibition

In a next step we decided to express some of the V_HHs into mammalian cells. Thereto COS1-B1 cells, stably expressing low levels of BACE1, were co-transfected with APPSw and either Nb_B26 or Nb_B9. Control cells were either transfected with empty vector or with APPSw alone. The cDNAs (of Nb_B26 or Nb_B9) were cloned into an eukaryotic expression vector, downstream of the signal peptide of BACE1 and with a myc-epitope tag at its C-terminus (**Fig. 3A**). The signal sequence ensures translocation of the newly formed protein into the secretory pathway, where the V_HH should encounter its antigen, the ectodomain of BACE1. APP_{Sw} and the 2 V_HHs were co-transfected into COS cells stably expressing low levels of human BACE1 (COS-B1 cells). These cells have detectable but not saturated levels of β -secretase activity and are easily transfected using liposome-based transfection reagents. Two days after transfection, cell extracts were prepared, proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Using rabbit polyclonal B63.1 as a primary antibody and GARIR, an infrared-coupled secondary antibody, the APP C-terminal fragments were visualized and quantified by the Odyssey Infrared Imaging System (**Fig. 3B & C**). Again, Nb_B26 had no effect on APP processing. The ratio of β -CTF on total APP CTFs is equal to that of non-treated cells (**Fig. 3C**). Nb_B9 consistently decreased β -secretase activity by about 30% ($p < 0,001$), even though it was expressed at much lower levels than Nb_B26 (**Fig. 3B**). This decrease occurred in the absence of any effect on BACE1 protein levels, ruling out the possibility that the nanobody affects BACE1 protein stability.

6. Effect of addition of extracellular nanobodies on APP processing in cells

In a next step Nanobody Nb_B9 was tested whether it could also affect APP processing when added to culture medium of cells. At least part of BACE1 is directed to the plasma membrane before being targeted to endosomes, so BACE1-specific antibodies could potentially bind to the ectodomain at the cell surface and be smuggled inside cells by co-internalization with their antigen. Since β -secretase cleavage of wild type APP predominantly occurs within the endosomal compartments, neutralizing the enzyme's activity from the plasma membrane on

might be sufficient to decrease β -site APP proteolysis. SH-SY5Y cells, neuroblastoma cells with relatively high endogenous BACE1 activity, were infected with recombinant adenoviruses containing the cDNA encoding either human APP wild type or the FAD APP_{sw} mutant. The FAD mutant was included since it is a much better BACE1 substrate, which enables easier
5 detection of A β and β -CTF. However, the majority of APP_{sw} has been shown to be cleaved at the β -site in the secretory pathway before reaching the plasma membrane (Martin et al., 1995, Thinakaran et al., 1996), so BACE1-neutralizing V_HHs binding at the cell surface might not be capable of preventing β -site APP_{sw} cleavage. Two days after adenoviral infection, the SH-SY5Y cells were radioactively labelled and incubated with 2 μ M Nb_B9 for 6 hours. The
10 conditioned medium was used to immunoprecipitate secreted A β , whereas APP full length and C-terminal fragments were pulled down from cell lysates. APP fragments were separated by SDS-PAGE. Gels were fixed, dried and analyzed by phosphorimaging. The presence of Nb_B9 caused clear detectable change in amounts of β -CTF or A β compared to non-treated cells (**Fig. 4**).

15 7. Isolation of other BACE1-specific nanobodies

Further, a new screening of the V_HH phage libraries was performed using a different panning strategy, while Nb_B9 was included for further analysis. Phage pannings of the two V_HH libraries were performed using biotinylated antigen (the ectodomain of human BACE1). After
20 three rounds of consecutive panning, 500 single colonies were randomly picked for phage ELISA screening. 158 out of 500 colonies scored positive in phage ELISA screening. The positive colonies were further screened by periplasmic extract ELISA, 44 colonies out the 158 colonies were scored positive. The positive colonies isolated from periplasmic extract ELISA screening were analyzed by PCR and restriction enzyme digestion to group them according to
25 restriction pattern and for further sequencing analysis. 14 new V_HHs were identified from the screening.

The alignment of the V_HHs sequence was listed in **Fig. 5**. Among these V_HHs, 10 clones 1B3, 10C2, 12B6 10B5, 13A5, 2C6, 6A4, 10C4, 13B6 and 1A4 (SEQ ID NOs: 15-24, respectively) were isolated from the dromedary libraries, and 4 clones 2B6, 4A2, 1D4 and 9D3 (SEQ ID
30 NOs: 25-28, respectively) were isolated from the llama library. The cDNA of these clones were subcloned into expression vector pHEN6 and V_HH antibodies were then purified for functional assay tests.

8. BACE1-specific nanobodies inhibit BACE1 activity in a cellular assay and modulate BACE1 activity in a cell-free enzymatic assay

All 15 V_HHs (14 new V_HHs + Nb_B9) were first tested in a cellular assay by adding them to the medium of SH-SY5Y cells stably expressing APP^{wt} at a final concentration of 20μM. As shown in **Fig. 6**, cells treated with V_HHs B9, 10C4, 4A2 for 24-hours were shown to decrease sAPPβ generation while sAPPα level in the conditioned medium remained the same as that of control, suggesting BACE1 activity was inhibited by these V_HHs in the cellular assay.

In parallel, the capacity of the V_HHs to modulate β-secretase activity was tested in an *in vitro* β-secretase assay that is based on the Fluorescence Resonance Energy Transfer (FRET)-technology. This assay makes use of a synthetic peptide substrate that mimics the BACE1 cleavage site of APP and is coupled to a fluorophore on its N-terminus and a fluorescence acceptor on its C-terminus. The light emitted by the fluorophore is absorbed by the fluorescence acceptor as long as these 2 moieties are in close proximity. Only upon proteolysis, when recombinant BACE1 is added to the synthetic substrate, energy transfer no longer occurs and the amount of light emitted, which is linearly related to the amount of cleaved product and hence to the β-secretase activity, can be measured. All V_HHs were tested by this BACE1 FRET assay at a final concentration of 10μM. As shown in **Fig. 7**, 10C4 and 4A2, the two candidate BACE1 inhibitors identified in the cellular assays, inhibited BACE1 activity in the FRET assay. Interestingly, B9, the candidate inhibitor isolated from cellular assay, was shown to increase 260% of BACE1 activity in FRET assay. Another clone, 1B3 was also shown to increase 125% of BACE1 activity in FRET assay, although it had no apparent effect on BACE1 in cellular assay. The remaining V_HHs had no or neglectable effects on BACE1 activity in the FRET assay.

The dose-response curves of 10C4, 4A2 and B9 on BACE1 activity were established by FRET assay. As shown in **Fig. 8**, 10C4 could inhibit maximal ~70% of BACE1 activity and the IC₅₀ was 150nM. 4A2 could inhibit maximal ~40% of BACE1 activity and the IC₅₀ was 1.2μM. B9 could increase BACE1 activity up to 3.5 times with ~100nM concentration, and the EC₅₀ in the response curve was 4,1nM.

The contradictory results from B9 modulating BACE1 activity in opposite ways in the cellular assay and the FRET assay implicates that B9 might have different effects on BACE1 cleavage of a large substrate or a small substrate (APP as the cellular substrate for BACE1 contains 695 amino acids while the peptide substrate in Fret assay contains only 10 amino acids). Therefore, it was tested whether B9 could inhibit BACE1 cleavage of a big substrate in another cell free enzymatic assay MBP-ELISA, which uses the maltose binding protein connected to the C-terminal 125 amino acids of APP^{swe} (MBP-APP^{swe}-C125) as BACE1 substrate. As

shown in **Fig. 9**, B9 inhibited BACE1 cleavage of MBP-APP_{swe}-C125 in a dose dependent manner, and could inhibit up to 95% of BACE1 activity. The results of this assay indicate that B9 is an inhibitor of BACE1 when using a big peptide substrate. So, V_HH Nb_B9, instead of being an active site binder, was more likely a steric inhibitor for BACE1. V_HH Nb_B9 could bind to an allosteric site on BACE1, thus stimulating BACE1 cleavage of small substrates that can still reach the cleavage site, but blocking access of big substrates, like APP to BACE1 by steric hindrance.

9. Affinity analysis of the BACE1-specific nanobodies

The binding affinities of V_HHs B9, 10C4 and 4A2 to human BACE1 ectodomain were analyzed by Biacore. As shown in **Table 2** (left), B9 had the best affinity among the three inhibitory V_HHs, with a K_d of 3,67nM at pH 7.0. 10C4 and 4A2 had affinities of 74.7nM and 48.2nM respectively, which are all within the normal range of affinities for V_HH antibodies.

Further, it was tested if the affinities of the V_HHs were stable at pH 4.5, at which BACE1 has its optimal activity. As shown in **Table 2** (right), there was no significant change in the affinities of the three V_HHs at pH 4.5 compared to that in neutral pH, indicating that all three V_HHs have binding affinities to BACE1 which were acidic stable.

Table 2. V_HH affinities to human BACE1 at pH 7.0 and pH 4.5

	pH 7.0			pH 4.5		
	k _{on}	k _{off}	K _D	k _{on}	k _{off}	K _D
	(M ⁻¹ s ⁻¹)	(s ⁻¹)	(nM)	(M ⁻¹ s ⁻¹)	(s ⁻¹)	(nM)
Nb_B9	2,67E+05	9,80E-04	3,67	6,62E+05	1,30E-03	1,96
Nb_4A2	4,79E+05	2,31E-02	48,2	3,97E+05	8,41E-03	21,2
Nb_10C4	1,06E+05	7,92E-03	74,7	4,51E+05	1,25E-02	27,7

The cross reactivity of three V_HHs to mouse BACE1 was investigated in anticipation of tests in primary cultures of mouse neurons. As shown in **Table 3**, both at neutral pH and acidic pH condition, all three V_HHs cross reacted with mouse BACE1, and their affinities to mouse BACE1 were within the same range of affinities as those measured with human BACE1.

Table 3. V_HH affinities to mouse BACE1 at pH 7.0 and pH 4.5

	pH 7.0			pH 4.5		
	k _{on}	k _{off}	K _D	k _{on}	k _{off}	K _D
	(M ⁻¹ s ⁻¹)	(s ⁻¹)	(nM)	(M ⁻¹ s ⁻¹)	(s ⁻¹)	(nM)
Nb_B9	5,02E+05	8,90E-04	1,77	1,06E+06	1,18E-03	1,11
Nb_4A2	1,65E+05	5,81E-03	35,2	2,51E+05	2,16E-03	8,61
Nb_10C4	1,90E+05	7,97E-03	41,9	9,84E+05	9,88E-03	10

10. BACE1-specific nanobodies inhibit BACE1 cleavage of APPwt in primary cultured mouse neurons

V_HHs Nb_B9 and Nb_4A2 were tested in the neuronal cell culture assay (**Figs. 10-11**). Primary cultured neurons from wild type mice were transduced with APPwt by Semliki Forest Virus (SFV), and then treated with purified V_HH Nb_B9 or Nb_4A2 by adding to the medium serial dilutions (V_HHs were first dissolved and diluted in PBS). Neuron cultures were metabolic labeled for 6 hours. CTFβ, sAPPβ and Aβ were later analyzed as readout of BACE1 activity. As shown in **Fig. 10**, Nb_B9 and Nb_4A2 inhibited BACE1 cleavage of APP reflected in the decrease of Aβ, sAPPβ, and CTFβ signals, while full length APP and sAPPα level remained at the same level as that of the control. The dose-response curve of Nb_B9 in neuron assay (**Fig. 11**) was established by quantification of the CTFβ level, which showed Nb_B9 inhibited BACE1 activity in a dose dependent manner, with maximal inhibition effect around 57% BACE1 activity and the IC50 was around 500nM.

11. Validation of BACE1-inhibitory nanobodies in mouse model

Camel single domain antibodies, the minimal sized antibodies, which have superior properties for intracellular expression and function, including solubility, stability and functionality without the requirement for association between heavy and light chains of conventional antibodies, are candidate therapeutic molecules for *in vivo* gene delivery. The BACE1 inhibitory V_HH Nb_B9 is tested in a transgenic mouse model of Alzheimer's disease through viral vector-mediated gene delivery. Adeno-associated virus (AAV), one of the most effective vehicles for gene delivery to central nervous system, is used in this experimentation. The cDNA of V_HH Nb_B9, fused with a

signal peptide from BACE1 at its N-terminal and a Myc-tag at its C-terminal, was constructed into an AAV vector. The AAV vector used here contains a hybrid cytomegalovirus/chicken β -actin promoter and a wood-chuck post-transcriptional regulatory element, which is an optimized cassette for driving protein expression in neurons (Björklund et al., 2000).

5 For *in vivo* testing, Dutch-mutant APP transgenic mice are used, which overexpress E693Q mutated human APP under the control of a neuron specific Thy1 promoter element (Herzig et al., 2004). The E693Q Dutch mutation site on human APP is 21 amino acid residues behind BACE1 cleavage site, which does not interfere with APP processing by BACE1. Transgenic mice overexpressing the Dutch-mutant APP generate predominantly A β 40 peptide, which is
10 used as readout for BACE1 activity for *in vivo* test of V_HH Nb_B9. Two administrative routes, including stereotactic injection to the hippocampus region of adult mouse brain (Fukuchi et al., 2006) and intracranial injection to neonatal mouse brain (Levites et al., 2006) are used for the delivery of AAV vector packaged V_HH Nb_B9. AAV vector packaged GFP and V_HH Nb_B24 are used as negative controls.

15 Materials and Methods

Cell culture

COS, BHK, MEF, CHO, HEK-APP_{sw}, N2A and HeLa cells were cultured at 37°C in a 5% CO₂ environment in Dulbecco's modified Eagle's medium / nutrient mixture F-12 (1:1) (Gibco)
20 supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Hyclone). The HEK-APP_{sw} cells were kindly provided by Prof. C. Haass (Adolf Butenandt Institute, Ludwig-Maximilians University, Munich, Germany). For transient liposome-based transfections, a mix of FuGENE 6 (Roche Applied Science) and plasmid DNA with a ratio of 3:1 (in μ l and μ g respectively) was added to a culture dish containing a 50-80% confluent monolayer of cells, according to the
25 manufacturer's instructions. COS-hBACE1 stable cells were obtained after transient transfection of COS cells with pcDNA3.1zeo-hBACE1 and selection in 400 μ g/ml zeocin (Invitrogen). SH-SY5Y cells were grown in DMEM GlutaMAX, 4500 mg/l D-glucose, 1 mM Sodium pyruvate (Gibco), supplemented with 15% (v/v) FBS.

Primary cortical neuronal cultures were isolated from E14 mouse embryos (according to Goslin
30 and Banker, 1991). Briefly, dissected brain cortices were trypsinized with 0.25% trypsin in HBSS medium (Gibco), pelleted and transferred to DMEM (Invitrogen, San Diego, CA) supplemented with 10% (v/v) FBS and dissociated by passing them through Pasteur pipettes of decreasing diameters. Dispersed cells were collected by centrifugation and plated on poly-L-lysine (Sigma)-coated dishes and maintained in neurobasal medium (Gibco) supplemented
35 with 0.5 μ M L-glutamine (Invitrogen) and 2% (v/v) B27 Serum-free Supplement (Gibco).

Cytosine arabinoside (5 μ M) was added 24 hours after plating to prevent proliferation of glial cells.

Metabolic labelling and immunoprecipitation of APP fragments

- 5 Cells were washed in Met-free or Met/Cys-free medium (GIBCO) and radioactively labelled in the appropriate medium containing respectively 100 μ Ci ^{35} S Met or ^{35} S Met/Cys (Trans ^{35} S Label, MP Biomedicals, Irvine, CA). In case of incubations with FK-506, rapamycin and Nb_B26 (2.1 μ M), compounds were added to the labelling medium. After 6 hours incubation, the culture supernatant was collected as a source of secreted A β or sAPP β and centrifuged to
10 remove detached cells. Cells were lysed in DIP buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium desoxycholate, 0.1 % SDS), except for the HEK-APP_{sw} cells, which were lysed in Tris buffered saline (TBS: 150 mM NaCl, 20 mM Tris-HCl, pH7.5), containing 1% Triton X-100 and a cocktail of protease inhibitors (Complete, Roche). This lysis buffer still allows to determine protein concentration (Bio-Rad Protein Assay) to analyze
15 efficiency of RNA interference on equal amounts of protein extract.

- APP full-length and APP C-terminal stubs were precipitated from cell extracts using the APP C-terminal antibodies B63.1, B11/4 or B12/6 (1:200) and immunocomplexes were captured by protein G-sepharose. For A β species, samples of the cell conditioned medium were incubated with either B7/8 or 4G8 (1:200). For the neurons overexpressing different BACE1 mutants,
20 BACE1 proteins were precipitated from cell extracts with B45.1.

- Immunoprecipitates were washed extensively in DIP buffer, followed by one washing step in TBS 1/3, eluted in LDS sample buffer (Invitrogen) supplemented with 1% β -mercapto ethanol and separated on 10 % NuPAGE gel (Novex) run in MES buffer for the APP fragments and MOPS for the BACE1 mutants. Gels were fixed, dried and exposed to a phosphorimaging
25 screen. Intensity of radioactive bands was quantified using PhosphorImaging (Typhoon, PerkinElmer) and the ImageQuant software package.

To detect sAPP β , samples of conditioned medium were subjected to SDS-PAGE and Western blotting using B53/4 antibody.

- 30 Deglycosylation experiments

Cells were harvested in Dulbecco's PBS (GIBCO), pelleted and lysed in 100 mM phosphate buffer at pH5.8 for EndoH treatment (46% of 0.2 M NaH₂PO₄, 4% of 0.2 M Na₂HPO₄ and 50% water) and pH7.4 for EndoF (9.5% of 0.2 M NaH₂PO₄, 40.5% of 0.2 M Na₂HPO₄ and 50% water), supplemented with 0.1% SDS, 0.5% Triton X-100, 0.5% β -mercapto-ethanol and

protease inhibitors (Complete, Roche). Lysates were first denatured by heating them for 10 minutes at 70°C and then treated with EndoH (1 unit/30 µl, Roche Applied Science) or EndoF (1 unit/30 µl, Roche Applied Science) for 19 hours at 37°C and analyzed by SDS-PAGE and Western blotting.

5

Generation of recombinant GST-fusion proteins

pGEX-4T-1 plasmids (Pharmacia) encoding GST fusion proteins were introduced in BL21 competent cells (Merck Eurolab) and expression of the GST proteins was induced by 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG, Promega). Recombinant proteins were released from the bacteria by sonication in a Tris-saline buffer (150 mM NaCl, 10 mM Tris) containing a protease inhibitor cocktail (1 mM EDTA, 14 µg/ml aprotinin, 2 µg/ml pepstatin), 100 µg/ml lysozyme, 5 mM DTT and 0.5% N-laurylsarcosine (sarcosyl) (Frangioni and Neel, 1993). After centrifugation at 12500 rpm (Beckman J2-21M/E), to remove insoluble bacterial debris, Triton X-100 was added to a final concentration of 1% to neutralize the effects of the ionic detergent sarcosyl.

15

Immunization of a dromedary and llama and analysis of the immune response

The immunization of dromedary and llama, the isolation of BACE1-binders and affinity measurements were done in collaboration with Prof. S. Muyldermans, VUB, Belgium.

20

With weekly intervals a dromedary and llama were immunized 6 times subcutaneously with 150 µg of pure recombinant human BACE1 mixed with GERBU adjuvant (GERBU Biochemicals). The immunogen used for the immunization of the dromedary was provided by Dr. S. Masure (Johnson & Johnson Pharmaceutical Research & Development, Beerse, Belgium). In order to obtain large amounts of active recombinant BACE1 insect cells were infected with baculoviruses encoding BACE1 ectodomain (sBACE1) in which the 4 putative N-glycosylation sites were removed by substituting the respective Asn codons for Gln codons (Bruinzeel et al., 2002). The lack of glycosylation made it possible to produce a large, homogeneous pool of BACE1.

25

A llama was immunized with a different source of BACE1. In this case the antigen was purified from sBACE1-overexpressing HEK293 cells (obtained from Prof. N. Mertens, Protein Service Facility, VIB, UGent) and hence resembled much better native, mature and thus fully glycosylated BACE1.

30

Forty-five days after the first injection anticoagulated blood was collected. BACE1 specific antibody titers for each IgG subclass were analyzed using ELISA. The 3 individual IgG subclasses were first purified from serum based on their differential absorption on Protein A

35

and Protein G and distinct elution conditions (Conrath et al., 2001a). Solid-phase coated BACE1 protein was incubated with serial dilutions of the different IgG subclasses and bound IgGs were subsequently detected with a rabbit anti-dromedary IgG antiserum and anti-rabbit IgG-alkaline phosphatase conjugates (Saerens et al., 2004).

5

Construction of a V_HH gene fragment library

Peripheral lymphocytes were isolated from the dromedary/llama sera (Lymphoprep, Nycomed) and total RNA was extracted (according to Chomczynski and Sacchi, 1987). After RT-PCR with a dN₆ primer, the cDNA obtained was used as template for the amplification of a DNA
10 fragment spanning the IgG variable domain until the CH2 domain, using primers CALL001 and CALL002 (see **Table 4**). These primers anneal to the IgG leader sequence and the CH2 exon of the heavy chain of all 3 IgG subclasses existing in dromedary, respectively. Using agarose gel extraction, the 600 bp fragment coming from heavy chain-only antibodies (V_HH-CH2, without CH1 domain) was separated from the 900 bp fragment derived from conventional
15 antibodies (V_H-CH1-CH2 exons). V_HH gene fragments were then amplified by PCR on the 600 bp DNA with a pair of nested primers, AE6 and FR4FOR (see **Table 4**). AE6 anneals to the V_HH framework-1 and contains a Pst I site, whereas FR4FOR with a Not I site is complementary to the framework-4. The different V_HH fragments were ligated into a pHEN4 phagemid vector and transformed into *E. coli* TG1 cells to create a library of 4x10⁷
20 transformants. Colony PCR screening showed that approximately 90% of the colonies were transformed with a phagemid vector containing an insert with the size expected for a V_HH fragment.

Table 4: Sequences of the different primers used for the V_HH gene fragment library
25 construction

Primer	Sequence (5' to 3')
CALL001	GTCCTGGCTGCTCTTCTACAAGG
CALL002	GGTACGTGCTGTTGAACTGTTCC
AE6	GATGTGCAGCTGCAGGAGTCTGGAGGAGG
FR4FOR	GGACTAGTGCGGCCGCTGCAGACGGTGACCTGGGT

Selection of BACE1- specific V_HH fragments

The V_HH repertoire was expressed onto the surface of phages after rescuing the library with M13K07 helper phages. Specific V_HHs against BACE1 were enriched by three consecutive rounds of *in vitro* selection, a technique also known as panning (Smith and Petrenko, 1997).

- 5 For this, the V_HHs were incubated on a solid phase coated with antigen. Unbound phages were washed away in PBS plus 0.05% Tween 20 and bound phages were eluted with 100 mM triethylamine (pH10.0). Eluted phage particles were immediately neutralized with 1 M Tris-HCl (pH7.5) and used to re-infect exponentially growing *E. coli* TG1 cells. After the second and third round of selection individual colonies were randomly picked.

10

Enzyme-linked Immunosorbent Assay (ELISA)

Expression of the selected V_HH was induced with 1 mM IPTG. The recombinant soluble C-terminally Hemagglutinin (HA)-tagged V_HHs (the gene encoding the HA-epitope is included in the pHEN4 phagemid vector) were extracted from the periplasm by an osmotic shock (200 mM

15 Tris-HCl pH8.0, 250 mM sucrose, 0.5 mM EDTA) (Skerra and Pluckthun, 1988) and tested for their capacity to recognize their antigen in ELISA tests. Maxisorb 96-well plates (Nunc) were coated overnight with BACE1 protein (100 µl of 1 µg/ml in PBS) at 4°C. Residual binding sites were blocked for 2 hours at room temperature with 1% (w/v) casein dissolved in PBS. This antigen coated solid phase was then incubated with the different periplasmic extracts for 1

20 hour at room temperature. After washing, the solid phase was successively incubated with mouse anti-HA, alkaline phosphatase-conjugated anti-mouse (Sigma) and 2 mg/ml *p*-nitrophenyl phosphate (Sigma). Signals were analyzed at 410 nm.

Expression and purification of V_HHs

- 25 The V_HH genes of the clones scoring positive in ELISA were subcloned into the expression vector pHEN6, using Pst I and BstE II. Thereby, the HA-epitope tag at the C-terminus of the V_HH molecules was replaced by a his6-tag. *E.coli* WK6 cells were transformed with the pHEN6 plasmids and expression of the recombinant soluble V_HH proteins was induced by IPTG (Saerens et al., 2004). Soluble V_HH molecules were extracted from the bacteria using an
- 30 osmotic shock (Skerra and Pluckthun, 1988). The his-tagged recombinant proteins were then captured on a nickel-nitrilotriacetic acid superflow Sepharose column (Qiagen), eluted with an acetate buffer (pH4.7), and additionally purified by size exclusion chromatography.

BIAcore measurements

The kinetic constants and affinity of the V_HH-antigen interactions were determined by surface plasmon resonance technology on a Biacore 3000 (Biacore AB). Purified V_HH molecules, in a concentration range of 0 - 500 nM in Hepes Buffered Saline pH7.0 or citrate buffer pH5.0, were injected at 30 µl/min onto BACE1 (500 resonance units), immobilized on a CM5 chip (according to De Genst et al., 2005). The kinetic and equilibrium constants (k_{on} , k_{off} and K_D) were determined with the BIAevaluation v3.1 software (Biacore AB).

In vitro FRET-based analysis of β -secretase activity

To determine whether the V_HHs affect BACE1 activity we used an *in vitro* BACE1 FRET assay kit (Panvera P2985). This assay uses a synthetic BACE1 substrate that emits light upon cleavage. The amount of total fluorescence is linearly related to the cleavage rate of the substrate and hence to β -secretase activity. Reaction mixtures containing 20 nM of recombinant BACE1 enzyme and 250 nM of synthetic substrate were incubated with an excess of each V_HH (2.2 µM) or the BACE1 inhibitor STA-200 (Enzyme System Products, 2.2 µM) in 50 mM sodium acetate, pH4.5 at room temperature, protected from light. After 2 hours, fluorescence was measured at 595 nm using VICTOR 1420 multilabel counter (Perkin Elmer Life Sciences). For each V_HH, the background signal, emitted by a mix containing V_HH and substrate but no enzyme, was subtracted from the signal measured for the mix containing V_HH, substrate and BACE1. As an alternative source of β -secretase activity, microsomal membranes were generated from HeLa cells ectopically expressing BACE1 as described herein before. The resulting microsomal pellet was resuspended in 50 mM sodium acetate, pH4.5. 50 µg of microsomal proteins were mixed with 250 nM of the synthetic BACE1 substrate and 2.2 µM of V_HH or STA-200. The enzymatic reaction and analysis were performed as before except that reactions were gently mixed every 10 min during the 2 hours-incubation.

In another approach, FRET peptide substrate MCA-S-E-V-N-L-D-A-E-F-R-K(Dnp)-R-R-R-R-NH₂ was synthesized by Ana Spec Inc (San Jose, CA, USA). Enzyme human BACE1 (1-460): IgGFc was purified from HEK293 cells according to the protocol described previously (Yang et al., 2004). For the reaction, enzyme was diluted in reaction buffer (50mM Ammonium Acetate, pH 4.6, 3% BSA, 0.7% TritonX-100) at concentration of 1µg/ml, and substrate was diluted in reaction buffer at concentration of 125µM. 20µl V_HH (diluted in reaction buffer) were mixed with 30µl enzyme dilution and 50µl substrate dilution in 96-well black polystyrene plates (Costar), the plates were read immediately for baseline signal with Envision (355nm excitation, 430nm emission, 1 sec/well), followed by incubation for overnight in dark at room temperature. The

plates were read the following morning using the same reader protocol, the Fret signal (-baseline signal) was used as the readout of enzyme activity in each reaction.

Co-precipitation of human BACE1 with his-tagged V_HHs using Ni-beads

- 5 BACE1-overexpressing COS cells were lysed in PBS containing 1% Triton X-100 and protease inhibitors (1 µg/ml pepstatin, 14µg/ml aprotinine, 0.5 mM pefabloc). 100µg of this protein extract were incubated overnight at 4°C with 2 µg of his-tagged V_HH proteins and Ni-PDC beads (Affiland) in binding buffer (342 mM NaCl, 16.2 mM Na₂HPO₄, 6.7 mM KCl, 3.7 mM KH₂PO₄ with 1% Triton X-100) with the same protease inhibitors as used for cell lysis. The
10 precipitates were washed in binding buffer supplemented with 10 mM imidazole, to reduce unspecific interactions, eluted using 300 µM imidazole and resolved by SDS-PAGE. BACE1 was visualized by Western blotting using a polyclonal rabbit anti-BACE1 antibody (ProSci Inc).

Phage libraries panning with biotin labeled antigen

- 15 Pannings of V_HH phage library were performed with biotin-streptavidin system. Purified BACE1 ectodomain protein was labeled with Sulfo-NHS-SS-Biotin (Pierce) according to manufacture's protocol. V_HH libraries were rescued with M13K07 helper phage to generate phages. For panning, 10¹¹ phages were blocked with 1% BSA in 400µl panning buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, and 0.05% Tween20) for 30min at RT. 100µl biotinylated BACE1 was
20 added to phage to a final concentration at 200nM. Phage and biotinylated BACE1 were incubated for 1 hour at RT with rotation. Meanwhile 40µl immobilized streptavidin (Pierce) were blocked in 1% BSA. After 1 hour incubation, pre-blocked immobilized streptavidin were added to phage-biotinylated BACE1 solution and incubated for 40min at RT with rotation. After incubation, immobilized streptavidin were spinned down by centrifugation at 3000 rpm for 1
25 min, the supernatant containing unbound phage were discarded. The immobilized streptavidin were washed 5 times with 1ml panning buffer, each wash lasted 5 min with rotation. After wash, 50mM DTT were added to immobilized streptavidin and incubated for 40min at RT with rotation. The immobilized streptavidin were spinned down and the supernatants containing eluted phages were used to re-infect *E. coli* TG1 cells for the next round phage panning. After
30 three rounds of consecutive panning, recovered phages were used to infect *E. coli* TG1 cells and plated out at 10⁻⁴ to 10⁻⁶ dilution, and single colonies were picked for further analysis.

Phage ELISA Screening

To generate phage particles with V_HHs displayed on the surface for ELISA screening, single colonies from phage panning were inoculated in 2 ml 2x TY medium supplemented with 50µg/ml ampicillin and 1% glucose in 24-well plates at 37°C for 8 hours with shaking at 220rpm. After 8 hours incubation, 5x 10⁸ pfu M13K07 helper phages were added to infect each well of bacterials. Infected bacterials were grown at 37°C overnight with shaking at 220rpm. The next morning, bacterials were spinned down by centrifugation at 3000 rpm for 20min, supernatants containing phage particles were transferred into 24-well plates. 20% PEG 6000/2.5M NaCl were added to the supernatants using 1/6 volume to precipitate phage particles at 4°C for 30min. Phage particles were later retrieved by centrifugation at 3000 rpm for 30 min, and pellets were resuspended in 100µl PBS.

For ELISA assay of phage particles, BACE1 ectodomain protein was coated on 48 wells of each 96-well microtiter plate at 100ng/well at 4°C overnight, the non-coated wells were used for control. The next morning, microtiter plates were blocked with 3% milk for 1 hour at RT. After blocking, 100µl phage particles were added to each coated and non-coated well, and were incubated for 2 hours at RT. Plates were then washed for 5 times with washing buffer (PBS, 0.05% Tween-20). After wash, HRP conjugated anti-M13 antibody (Amersham) was added to each well using 1:3000 dilution in 3% milk and incubated for 1 hour. Plates were then washed 5 times with washing buffer. After wash, developing substrate 0.02 mg/ml ABTS (Sigma) supplemented with 0.3% H₂O₂ (Sigma) were added to microtiter plates and incubated for 30min at RT. Plates were read at OD405 nm with a ELISA reader.

Periplasmic extract ELISA Screening

Expression vector pHEN4 containing a PelB (Pectate lysase) signal sequence before the V_HH cDNAs, thus V_HHs are exported to the periplasmic space after expressed in bacterial system. To generate periplasmic extract containing V_HH proteins for ELISA test, single colonies from phage panning were inoculated in 1ml Terrific Broth (TB) medium supplemented with 100µg/ml ampicillin in 24-well plates at 37°C with shaking at 220rpm. When OD₆₀₀ reach 0.6, 1mM IPTG were added to the culture to induce the expression of V_HH proteins. Bacterials were further incubated for 15 hours at 28°C for protein expression. After the incubation, bacterials were harvested by centrifugation at 3000 rpm for 20min, cell pellets were dissolved in TES solution (20mM Tris-HCl pH7.4, 1mM EDTA, 250mM sucrose) and incubated on ice for 30min. The osmotic shock was given by adding 1.5 x volume TES/4 to the bacterials and incubated on ice for 45min. Supernatants containing V_HH proteins were collected by centrifugation at 300rpm for 20min and further used for ELISA. The ELISA assays were followed the same protocol

described above for phage ELISA. To detect V_HH proteins generated with a C-terminal HA tag, anti-HA monoclonal antibody (Amersham) was used as primary antibody and alkaline phosphatase conjugated goat anti-mouse antibody (Amersham) were used as secondary antibody. ELISA plates were developed with p-Nitrophenyl-phosphate (PNPP) substrate (Sigma) and read at OD405nm with an ELISA reader.

Adeno-associated virus (AAV) construction and preparation

For AAV generation, a standard method was followed (Levites et al., 2006). Briefly, the cDNA of V_HH Nb_B9, fused with a signal peptide from BACE1 at its N-terminal and a Myc-tag at its C-terminal, was constructed into an AAV vector containing a hybrid cytomegalovirus/chicken β -actin promoter and a wood chuck post-transcriptional regulatory element. AAVs were generated by plasmid transfection with helper plasmids in HEK293T cells. Forty-eight hours after transfection, the cells were harvested and lysed in the presence of 0,5% sodium deoxycholate and 50U/ml Benzonase (Sigma) by freeze thawing, and the virus was isolated using a discontinuous iodixanol gradient purified on a HiTrap HQ column (Amersham Biosciences). The genomic titer of virus was determined by quantitative PCR.

Mice

All animal experiments were in compliance with protocols approved by the local Animal Care and Use Committees. Dutch-mutant APP transgenic mice (C57BL/6J-TgN(Thy-APP_{E693D})) were kindly provided by the laboratory of Mathias Jucker, University of Tübingen, Germany.

Stereotaxic injections

In the first series experiments, AAV vectors expressing V_HH Nb_B9 and GFP (negative control), V_HH Nb_B24 (negative control) were administrated directly into the hippocampus of 3-month-old Dutch-mutant APP transgenic mice. Mice were anesthetized with avertin and placed in a stereotaxic apparatus. AAV preparations were injected bilaterally (2 μ l per site) into the CA3 region of the hippocampus (-2.0 mm antero-posterior from bregma, +/- 2.3mm medio-lateral from bregma, and 1.7 mm below dura). Mice were then individually housed and allowed to recover from surgery. Their brains were processed for analyses 5 weeks after treatment.

Neonatal injections

The procedure was described previously (Levites et al., 2006). Briefly, postnatal day 0 (P0) pups were cryoanesthetized on ice for 5 min. AAV preparations (2 μ l) were injected intracerebroventricularly into both hemispheres using a 10ml Hamilton syringe with a 30 gauge

needle. The pups were then placed on a heating pad with their original nesting materials for 3-5 min and returned to their mother for further recovery. Their brains were processed for analyses 3 months after injection.

5 Tissue preparation and biochemical analysis of A β

To analyze A β , the hippocampus (from stereotaxic injections) and the whole brains (from neonatal injections) were homogenized in Tissue Protein Extraction reagent (Pierce) supplemented with complete protease inhibitor and phosphatase inhibitor tablets (Roche Applied Science). The homogenized samples were centrifuged at 4°C for 1 hour at 100,000 x
10 g, and the supernatant was used for immunoblot analysis and for A β ELISA measurements using ELISA kits (The Genetics Company).

References

- Arbabi Ghahroudi, M., Desmyter, A., Wyns, L., Hamers, R. and Muyldermans, S., 1997. Selection and identification of single domain antibody fragments from camel heavy-chain antibodies. *FEBS Lett.* 414, 521-526.
- 5 Björklund A, Kirik D, Rosenblad C, Georgievska B, Lundberg C, Mandel RJ. 2000. Towards a neuroprotective gene therapy for Parkinson's disease: use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model. *Brain Res.* 886, 82-98.
- 10 Bruinzeel, W., Yon, J., Giovannelli, S. and Masure, S., 2002. Recombinant insect cell expression and purification of human beta-secretase (BACE-1) for X-ray crystallography. *Protein Expr Purif.* 26, 139-148.
- Chomczynski, P. and Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem.* 162, 156-159.
- 15 Conrath, K. E., Lauwereys, M., Galleni, M., Matagne, A., Frere, J. M., Kinne, J., Wyns, L. and Muyldermans, S., 2001a. Beta-lactamase inhibitors derived from single-domain antibody fragments elicited in the camelidae. *Antimicrob Agents Chemother.* 45, 2807-2812.
- Conrath, K. E., Wernery, U., Muyldermans, S. and Nguyen, V. K., 2003. Emergence and evolution of functional heavy-chain antibodies in Camelidae. *Dev Comp Immunol.* 27, 87-103.
- 20 De Genst, E., Silence, K., Ghahroudi, M. A., Decanniere, K., Loris, R., Kinne, J., Wyns, L. and Muyldermans, S., 2005. Strong in vivo maturation compensates for structurally restricted H3 loops in antibody repertoires. *J Biol Chem.* 280, 14114-14121.
- 25 Desmyter, A., Transue, T. R., Ghahroudi, M. A., Thi, M. H., Poortmans, F., Hamers, R., Muyldermans, S. and Wyns, L., 1996. Crystal structure of a camel single-domain VH antibody fragment in complex with lysozyme. *Nat Struct Biol.* 3, 803-811.
- Frangioni, J. V. and Neel, B. G., 1993. Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. *Anal Biochem.* 210, 179-187.
- 30 Fukuchi K, Tahara K, Kim HD, Maxwell JA, Lewis TL, Accavitti-Loper MA, Kim H, Ponnazhagan S, Lalonde R. 2006. Anti-Abeta single-chain antibody delivery via adeno-associated virus for treatment of Alzheimer's disease. *Neurobiol Dis.* 23, 502-11.
- Goslin, K. and Banker, G., 1991. Rat hippocampal neurons in low-density culture. *In* *Culturing Nerve Cells*. MIT Press, Cambridge, Massachusetts.

- Herzig MC, Winkler DT, Burgermeister P, Pfeifer M, Kohler E, Schmidt SD, Danner S, Abramowski D, Stürchler-Pierrat C, Bürki K, van Duinen SG, Maat-Schieman ML, Staufenbiel M, Mathews PM, Jucker M. 2004. Abeta is targeted to the vasculature in a mouse model of hereditary cerebral hemorrhage with amyloidosis. *Nat Neurosci.* 7, 954-60.
- Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S. and Foeller, C., 1991. Sequences of proteins of immunological interest. US Public Health Services, NIH, Bethesda, MD.
- Koo, E. H. and Squazzo, S. L., 1994. Evidence that production and release of amyloid beta-protein involves the endocytic pathway. *J Biol Chem.* 269, 17386-17389.
- Lauwereys, M., Arbabi Ghahroudi, M., Desmyter, A., Kinne, J., Holzer, W., De Genst, E., Wyns, L. and Muyldermans, S., 1998. Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. *Embo J.* 17, 3512-3520.
- Lesk, A. M. and Chothia, C., 1988. Elbow motion in the immunoglobulins involves a molecular ball-and-socket joint. *Nature.* 335, 188-190.
- Levites Y, Jansen K, Smithson LA, Dakin R, Holloway VM, Das P, Golde TE. 2006. Intracranial adeno-associated virus-mediated delivery of anti-pan amyloid beta, amyloid beta40, and amyloid beta42 single-chain variable fragments attenuates plaque pathology in amyloid precursor protein mice. *J Neurosci.* 26, 11923-8.
- Lin, X., Koelsch, G., Wu, S., Downs, D., Dashti, A. and Tang, J., 2000. Human aspartic protease memapsin 2 cleaves the beta-secretase site of beta-amyloid precursor protein. *Proc Natl Acad Sci U S A.* 97, 1456-1460.
- Martin, B. L., Schrader-Fischer, G., Busciglio, J., Duke, M., Paganetti, P. and Yankner, B. A., 1995. Intracellular accumulation of beta-amyloid in cells expressing the Swedish mutant amyloid precursor protein. *J Biol Chem.* 270, 26727-26730.
- Muyldermans, S., Atarhouch, T., Saldanha, J., Barbosa, J. A. and Hamers, R., 1994. Sequence and structure of VH domain from naturally occurring camel heavy chain immunoglobulins lacking light chains. *Protein Eng.* 7, 1129-1135.
- Muyldermans, S. and Lauwereys, M., 1999. Unique single-domain antigen binding fragments derived from naturally occurring camel heavy-chain antibodies. *J Mol Recognit.* 12, 131-140.
- Nguyen, V. K., Hamers, R., Wyns, L. and Muyldermans, S., 2000. Camel heavy-chain antibodies: diverse germline V(H)H and specific mechanisms enlarge the antigen-binding repertoire. *Embo J.* 19, 921-930.
- Padlan, E. A., 1994. Anatomy of the antibody molecule. *Mol Immunol.* 31, 169-217.

Saerens, D., Kinne, J., Bosmans, E., Wernery, U., Muyldermans, S. and Conrath, K., 2004. Single domain antibodies derived from dromedary lymph node and peripheral blood lymphocytes sensing conformational variants of prostate-specific antigen. *J Biol Chem.* 279, 51965-51972.

- 5 Sinha, S., Anderson, J. P., Barbour, R., Basi, G. S., Caccavello, R., Davis, D., Doan, M., Dovey, H. F., Frigon, N., Hong, J., Jacobson-Croak, K., Jewett, N., Keim, P., Knops, J., Lieberburg, I., Power, M., Tan, H., Tatsuno, G., Tung, J., Schenk, D., Seubert, P., Suomensari, S. M., Wang, S., Walker, D., John, V. and et al., 1999. Purification and cloning of amyloid precursor protein beta-secretase from human brain. *Nature.* 402, 537-540.
- 10

Skerra, A. and Pluckthun, A., 1988. Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science.* 240, 1038-1041.

Smith, G. P. and Petrenko, V. A., 1997. Phage Display. *Chem Rev.* 97, 391-410.

- Thinakaran, G., Teplow, D. B., Siman, R., Greenberg, B. and Sisodia, S. S., 1996. Metabolism of the "Swedish" amyloid precursor protein variant in neuro2a (N2a) cells. Evidence that cleavage at the "beta-secretase" site occurs in the golgi apparatus. *J Biol Chem.* 271, 9390-9397.
- 15

- Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M. A., Biere, A. L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Treanor, J., Rogers, G. and Citron, M., 1999. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science.* 286, 735-741.
- 20

- Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., Brashier, J. R., Stratman, N. C., Mathews, W. R., Buhl, A. E., Carter, D. B., Tomasselli, A. G., Parodi, L. A., Heinrikson, R. L. and Gurney, M. E., 1999. Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. *Nature.* 402, 533-537.
- 25

$\langle 210 \rangle$	2
$\langle 211 \rangle$	127

41b

<212> PRT

<213> Camelus dromedarius

<400> 2

Asp	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Ser	Val	Gln	Ala	Gly	Gly
1				5					10					15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Val	Ser	Gly	Phe	Ser	Tyr	Ser	Pro	Tyr
			20					25					30		
Tyr	Met	Gly	Trp	Phe	Arg	Gln	Ala	Pro	Gly	Lys	Glu	Arg	Glu	Gly	Val
		35					40					45			
Ala	Ala	Ile	Arg	Lys	Gly	Ile	Gly	Thr	Thr	Tyr	Tyr	Ala	Asp	Ser	Val
	50					55					60				
Lys	Gly	Arg	Phe	Thr	Phe	Ser	Gln	Asp	Asp	Ala	Lys	Asn	Thr	Met	Tyr
65					70				75					80	
Leu	Gln	Met	Asn	Ser	Leu	Lys	Pro	Glu	Asp	Thr	Ala	Ile	Tyr	Phe	Cys
			85					90						95	
Ala	Val	Gly	His	Tyr	Arg	Ala	Tyr	Ala	Thr	Thr	Ser	Phe	Asp	Pro	Arg
			100					105					110		
Arg	Tyr	Asp	Tyr	Trp	Gly	Gln	Gly	Thr	Gln	Val	Thr	Val	Ser	Ser	
		115					120					125			

<210> 3

<211> 125

<212> PRT

<213> Camelus dromedarius

<400> 3

Asp	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Ser	Val	Gln	Ala	Gly	Gly
1				5					10					15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Val	Ser	Gly	Tyr	Thr	Tyr	Asn	Ile	Tyr
			20					25					30		
Thr	Met	Ala	Trp	Phe	Arg	Gln	Ala	Pro	Gly	Lys	Glu	Arg	Glu	Gly	Val
		35					40					45			
Ala	Gly	Ile	Tyr	Ser	Pro	Gly	Gly	Thr	Thr	Tyr	Tyr	Ala	Asp	Ser	Val
	50					55					60				
Lys	Gly	Arg	Phe	Thr	Ile	Ser	Gln	Asp	Asn	Ala	Lys	Asn	Thr	Val	Tyr
65					70				75					80	
Leu	Gln	Met	Asn	Ser	Leu	Ala	Pro	Glu	Asp	Thr	Ala	Ile	Tyr	Tyr	Cys
			85					90						95	
Ala	Ala	Arg	Gly	Gly	Leu	Leu	Ser	Arg	Val	Leu	Lys	Glu	Ala	Gly	Tyr
			100					105					110		
Asn	Ala	Trp	Gly	Gln	Gly	Thr	Gln	Val	Thr	Val	Ser	Ser			
		115					120					125			

<210> 4

<211> 125

<212> PRT

<213> Camelus dromedarius

<400> 4

Asp	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Ser	Val	Gln	Ala	Gly	Gly
1				5					10					15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Tyr	Thr	Phe	Thr	Lys	Tyr
			20					25					30		
Pro	Met	Gly	Trp	Phe	Arg	Gln	Ala	Pro	Gly	Lys	Glu	Cys	Glu	Leu	Val
		35					40					45			
Ser	Ser	Ile	Ile	Ser	Gly	Gly	Val	Thr	Thr	Tyr	Ala	Ser	Ser	Val	Lys
	50					55					60				

41c

Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn	Thr	Val	Tyr	Leu
65					70					75					80
Gln	Met	Asn	Ser	Leu	Lys	Pro	Glu	Asp	Thr	Ala	Val	Tyr	Tyr	Cys	Ala
				85					90					95	
Ala	Gln	Tyr	Pro	Tyr	Ser	Ser	Ser	Trp	Pro	Arg	Cys	Pro	Phe	Arg	Ile
			100					105					110		
Gly	Tyr	Trp	Gly	Gln	Gly	Thr	Gln	Val	Thr	Val	Ser	Ser			
		115					120					125			

<210> 5
 <211> 123
 <212> PRT
 <213> Camelus dromedarius

<400> 5

Asp	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Ser	Val	Gln	Ala	Gly	Gly
1				5					10					15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Arg	Ser	Gly	Gly	Thr	Val	Ser	Ile	Pro
			20					25					30		
Tyr	Met	Ala	Trp	Phe	Arg	Gln	Gly	Pro	Gly	Lys	Glu	Arg	Glu	Gly	Val
		35					40					45			
Ala	Ala	Ile	Tyr	Asp	Gly	Arg	Ala	Lys	Thr	Tyr	Ala	Gly	Ser	Leu	Gln
	50					55					60				
Gly	Arg	Phe	Thr	Ile	Ser	Gln	Asp	Asn	Asp	Lys	Asn	Thr	Leu	Tyr	Leu
65					70					75					80
Gln	Met	Asn	Ser	Leu	Lys	Pro	Asp	Asp	Thr	Ala	Val	Tyr	Tyr	Cys	Ala
				85				90						95	
Ala	Gly	Asn	Gly	Gly	Gly	Asn	Trp	Leu	Arg	Pro	Ser	Glu	Tyr	Asn	Tyr
			100					105					110		
Trp	Gly	Lys	Gly	Thr	Gln	Val	Thr	Val	Ser	Ser					
		115					120								

<210> 6
 <211> 121
 <212> PRT
 <213> Camelus dromedarius

<400> 6

Asp	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Ser	Val	Gln	Ala	Gly	Gly
1				5					10					15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Glu	Tyr	Thr	Tyr	Gly	Tyr	Cys
			20					25					30		
Ser	Met	Gly	Trp	Tyr	Arg	Gln	Ala	Pro	Gly	Lys	Glu	Arg	Glu	Leu	Val
		35					40					45			
Ser	Thr	Ile	Thr	Ser	Asp	Gly	Ser	Thr	Ser	Tyr	Val	Asp	Ser	Val	Lys
	50					55					60				
Gly	Arg	Phe	Thr	Ile	Ser	Gln	Asp	Asn	Ala	Lys	Asn	Thr	Val	Tyr	Leu
65					70					75					80
Gln	Met	Asn	Ser	Leu	Lys	Pro	Glu	Asp	Thr	Ala	Lys	Tyr	Tyr	Cys	Tyr
				85				90						95	
Thr	Lys	Thr	Cys	Ala	Asn	Lys	Leu	Gly	Ala	Lys	Phe	Ile	Ser	Trp	Gly
			100					105					110		
Gln	Gly	Thr	Gln	Val	Thr	Val	Ser	Ser							
		115					120								

<210> 7
 <211> 127

41d

<212> PRT
 <213> Camelus dromedarius

<400> 7
 Asp Val Gln Leu Gln Glu Ser Gly Gly Gly Ser Val Gln Pro Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Phe Tyr Ser Arg Trp
 20 25 30
 Tyr Met Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val
 35 40 45
 Ala Ala Ile Asn Ser Gly Gly Ser Ile Thr Ser Tyr Ala Asp Ser Val
 50 55 60
 Lys Gly Arg Phe Thr Ile Ser Gln Asp Asn Ala Lys Asn Thr Val Tyr
 65 70 75 80
 Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Ile Tyr Tyr Cys
 85 90 95
 Ala Ala Ala Leu Ser Arg Val Pro Gly Phe Phe Pro Leu Phe Pro Ser
 100 105 110
 Gln Tyr Asn Tyr Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser
 115 120 125

<210> 8
 <211> 129
 <212> PRT
 <213> Camelus dromedarius

<400> 8
 Asp Val Gln Leu Gln Glu Ser Gly Gly Gly Ser Ala Gln Ala Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Glu Val Ser Gly Tyr Thr Tyr Ser Gly Tyr
 20 25 30
 Phe Met Gly Trp Phe Arg Gln Ala Pro Gly Glu Glu Arg Glu Gly Val
 35 40 45
 Ala Ala Ile Asp Thr Asn Gly Gly Arg Thr Trp Tyr Ala Asp Ser Val
 50 55 60
 Lys Gly Arg Phe Thr Ile Ser His Asp Asn Ala Glu Ser Thr Val Tyr
 65 70 75 80
 Leu Gln Met Asn Ser Leu Gln Pro Glu Asp Thr Ala Ile Tyr Phe Cys
 85 90 95
 Ala Ala Arg Arg Pro Pro Gly Gly Ser Trp Tyr Pro Pro Pro Leu Arg
 100 105 110
 Lys Tyr Ser Tyr Asn Phe Trp Gly Gln Gly Thr Gln Val Thr Val Ser
 115 120 125
 Ser

<210> 9
 <211> 128
 <212> PRT
 <213> Camelus dromedarius

<400> 9
 Asp Val Gln Leu Gln Glu Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Val Ala Ser Gly Phe Thr Tyr Arg Arg Tyr
 20 25 30
 Phe Met Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Ala Val
 35 40 45

41e

Ala	Thr	Met	Phe	Ser	Cys	Gly	Gly	Thr	Thr	Tyr	Tyr	Ala	Asp	Ser	Val
50						55						60			
Lys	Gly	Arg	Phe	Thr	Ala	Thr	Gln	Asp	Asn	Ala	Lys	Asn	Thr	Val	Tyr
65					70					75					80
Leu	Gln	Met	Asn	Asn	Leu	Lys	Pro	Glu	Asp	Thr	Ala	Ile	Tyr	Tyr	Cys
				85					90					95	
Ala	Ala	Ala	Ser	Gly	Cys	Trp	Tyr	Asp	Gly	Ser	Pro	Ala	Ala	Arg	Ser
			100					105					110		
Val	Asp	Val	Ser	Phe	Trp	Gly	His	Gly	Thr	Gln	Val	Thr	Val	Ser	Ser
		115					120					125			

<210> 10
 <211> 129
 <212> PRT
 <213> Camelus dromedarius

<400> 10

Asp	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Ser	Val	Gln	Ala	Gly	Gly
1				5					10					15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Tyr	Ser	Tyr	Ser	Tyr	Tyr
			20					25					30		
Ile	Gly	Trp	Phe	Arg	Gln	Ala	Pro	Gly	Lys	Glu	Arg	Glu	Gly	Val	Ala
	35					40						45			
Ala	Ile	Ala	Ile	Val	Asn	Ser	Gly	Gly	Gly	Arg	Thr	Tyr	Tyr	Ala	Asp
	50					55					60				
Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Gln	Gly	Asn	Asp	Lys	Asn	Thr
65					70					75					80
Val	Tyr	Leu	Gln	Met	Asn	Ser	Leu	Lys	Pro	Glu	Asp	Thr	Ala	Ile	Tyr
				85					90					95	
Tyr	Cys	Ala	Ala	Arg	Ser	Leu	Ser	Trp	Tyr	Ser	His	Pro	Leu	Leu	Gln
			100					105					110		
Pro	Ser	Gln	Phe	Asn	Asn	Trp	Gly	Gln	Gly	Thr	Gln	Val	Thr	Val	Ser
		115					120					125			

Ser

<210> 11
 <211> 128
 <212> PRT
 <213> Camelus dromedarius

<400> 11

Asp	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Ser	Val	Gln	Ala	Glu	Gly
1				5					10					15	
Ser	Leu	Arg	Leu	Ser	Cys	Thr	Ala	Ser	Gly	Tyr	Thr	Tyr	Ser	Leu	Met
			20					25					30		
Gly	Trp	Phe	Arg	Gln	Ala	Pro	Gly	Lys	Glu	Arg	Glu	Gly	Val	Ala	Val
	35					40						45			
Ile	Asn	Ser	Gly	Val	Gly	Thr	Thr	Tyr	Tyr	Ala	Asp	Ser	Val	Lys	Gly
	50					55					60				
Arg	Phe	Thr	Ile	Ser	Gln	Asp	Asn	Ala	Lys	Ser	Thr	Val	Tyr	Leu	Gln
65					70					75					80
Met	Asn	Ser	Leu	Lys	Pro	Glu	Asp	Thr	Ala	Ile	Tyr	Tyr	Cys	Ala	Ala
				85					90					95	
Arg	Arg	Ser	Trp	Phe	Thr	Gly	Met	Thr	Thr	Thr	Gln	Ala	Leu	Asp	Pro
			100					105					110		
Asp	Trp	Phe	Ser	Tyr	Trp	Gly	Gln	Gly	Thr	Gln	Val	Thr	Val	Ser	Ser
		115					120					125			

41f

<210> 12
 <211> 125
 <212> PRT
 <213> Camelus dromedarius

<400> 12
 Asp Val Gln Leu Gln Glu Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Ser Glu Met Asn
 20 25 30
 Arg Phe Ala Trp Leu Arg Gln Ala Pro Gly Lys Asp Arg Glu Val Val
 35 40 45
 Ala Val Ile Phe Pro Thr Ala Arg Gly Ala Lys Phe Tyr Ser Asp Ser
 50 55 60
 Val Asn Gly Arg Phe Thr Ile Ser Gln Asp Thr Ala Lys Asn Thr Val
 65 70 75 80
 Tyr Leu Gln Met Asn Ser Leu Glu Pro Glu Asp Thr Ala Met Tyr Phe
 85 90 95
 Cys Ala Ala Ser Ala Asn Ala Met Thr Gly Phe Gln Pro Ser Gly Tyr
 100 105 110
 Thr Tyr Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser
 115 120 125

<210> 13
 <211> 129
 <212> PRT
 <213> Camelus dromedarius

<400> 13
 Gln Val Gln Leu Gln Glu Ser Gly Gly Gly Thr Val Gln Ala Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Tyr Arg Ser Tyr
 20 25 30
 Cys Met Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Glu Val
 35 40 45
 Ala Ser Ile Asn Ser Asp Gln Gly Ser Thr Arg Tyr Ala Ala Ser Val
 50 55 60
 Lys Gly Arg Phe Thr Ser Ser Gln Asp Asn Ala Asn Asn Thr Val Thr
 65 70 75 80
 Val Tyr Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Ile Tyr
 85 90 95
 Tyr Cys Ala Ala Asn Asp Gly Cys Ala Tyr Arg Val Tyr Arg Gly Gly
 100 105 110
 Ala Tyr Gly Tyr Asn Phe Trp Gly Gln Gly Thr Gln Val Thr Val Ser
 115 120 125
 Ser

<210> 14
 <211> 129
 <212> PRT
 <213> Camelus dromedarius

<400> 14
 Asp Val Gln Leu Gln Glu Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ala Phe Ser Ser Tyr
 20 25 30

41g

Tyr Met Gly Trp Phe Arg Gln Ala Pro Gly Arg Glu Arg Glu Glu Val
 35 40 45
 Thr Gly Ile Thr Gln Ile Gly Gly Thr Thr Tyr Tyr Ala Asp Ser Val
 50 55 60
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr
 65 70 75 80
 Leu Gln Met Asp Ser Leu Lys Pro Glu Asp Thr Ala Ile Tyr Tyr Cys
 85 90 95
 Ala Lys Leu Arg Arg Pro Phe Tyr Tyr Pro Leu Leu Glu Arg Pro Ser
 100 105 110
 Glu Gly Asp Phe Asp Tyr Trp Gly Gln Gly Thr Gln Val Thr Val Ser
 115 120 125
 Ser

<210> 15
 <211> 126
 <212> PRT
 <213> Camelus dromedarius

<400> 15
 Gln Val Gln Leu Gln Glu Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Glu Tyr Thr Asp Ser Thr Tyr
 20 25 30
 Tyr Met Ala Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Gly Gly Val
 35 40 45
 Ala Thr Leu Ala Ser Arg Tyr Asp Thr Thr Tyr Tyr Ala Asp Ser Val
 50 55 60
 Lys Gly Arg Phe Thr Ile Ser Gln Asp Arg Ala Lys Asn Thr Val Tyr
 65 70 75 80
 Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Gly Ile Tyr Tyr Cys
 85 90 95
 Ala Ala Ser Pro Arg Arg Pro Gly Phe Phe Pro Leu Asp Pro Ser Gln
 100 105 110
 Tyr Asn Tyr Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser
 115 120 125

<210> 16
 <211> 127
 <212> PRT
 <213> Camelus dromedarius

<400> 16
 Gln Val Gln Leu Gln Glu Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Arg Asp Ile Leu Thr Leu Tyr
 20 25 30
 Tyr Met Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val
 35 40 45
 Ala Ala Ile Ser Ser Asp Ile Ile Phe Thr Ser Tyr Ala Asn Ser Val
 50 55 60
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Lys Asp Lys Asn Thr Val Tyr
 65 70 75 80
 Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Ile Tyr Tyr Cys
 85 90 95
 Ala Ala Ala Ser Thr Trp Val Pro Gly Phe Phe Pro Leu Phe Ala Ser
 100 105 110

41h

Gln Tyr Asn Ser Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser
 115 120 125

<210> 17
 <211> 127
 <212> PRT
 <213> Camelus dromedarius

<400> 17
 Gln Val Gln Leu Gln Glu Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Arg Asp Ile Leu Thr Leu Tyr
 20 25 30
 Tyr Met Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val
 35 40 45
 Ala Ala Ile Ser Ser Asp Ile Ile Phe Thr Ser Tyr Ala Asn Ser Val
 50 55 60
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Glu Asp Lys Asn Thr Val Tyr
 65 70 75 80
 Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Ile Tyr Tyr Cys
 85 90 95
 Ala Ala Ala Ser Thr Trp Val Pro Gly Phe Phe Pro Leu Phe Ala Ser
 100 105 110
 Gln Tyr Asn Ser Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser
 115 120 125

<210> 18
 <211> 125
 <212> PRT
 <213> Camelus dromedarius

<400> 18
 Gln Val Gln Leu Gln Glu Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala His Ser Asn Thr Tyr Pro Thr Tyr Met
 20 25 30
 Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val Ala Ala
 35 40 45
 Ile Tyr Thr Gly Asp Gly Thr Thr Tyr Tyr Gly Asp Ser Val Lys Gly
 50 55 60
 Arg Phe Thr Ile Ser Gln Asp Asn Ala Lys Asn Thr Val Tyr Leu Gln
 65 70 75 80
 Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Ile Tyr Tyr Cys Ala Ala
 85 90 95
 Ala Leu Ser Arg Val Pro Gly Phe Phe Pro Leu Phe Pro Ser Gln Tyr
 100 105 110
 Asn Tyr Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser
 115 120 125

<210> 19
 <211> 125
 <212> PRT
 <213> Camelus dromedarius

<400> 19
 Gln Val Gln Leu Gln Glu Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
 1 5 10 15

41i

Ser	Leu	Arg	Leu	Ser	Cys	Ala	His	Ser	Asn	Thr	Tyr	Pro	Thr	Tyr	Met
			20					25					30		
Gly	Trp	Phe	Arg	Gln	Ala	Pro	Gly	Lys	Glu	Arg	Glu	Gly	Val	Ala	Ala
		35					40					45			
Ile	Tyr	Thr	Gly	Asp	Gly	Thr	Thr	Tyr	Tyr	Gly	Asp	Ser	Val	Lys	Gly
	50					55					60				
Arg	Phe	Thr	Ile	Ser	Gln	Asp	Asn	Ala	Lys	Asn	Thr	Val	Tyr	Leu	Gln
65					70					75					80
Met	Asn	Ser	Leu	Lys	Pro	Glu	Asp	Thr	Ala	Ile	Tyr	Tyr	Cys	Ala	Ala
				85					90					95	
Ala	Leu	Ser	Arg	Val	Pro	Gly	Phe	Phe	Pro	Leu	Phe	Pro	Ser	Gln	Tyr
			100					105					110		
Asn	Tyr	Trp	Gly	Gln	Gly	Thr	Gln	Val	Thr	Val	Ser	Ser			
		115					120					125			

<210> 20
 <211> 127
 <212> PRT
 <213> Camelus dromedarius

<400> 20															
Gln	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Ser	Val	Gln	Ala	Gly	Gly
1			5					10						15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Ser	Ser	Val	Tyr
			20					25					30		
Tyr	Ile	Ser	Trp	Phe	Arg	Gln	Ala	Pro	Gly	Lys	Glu	Arg	Glu	Gly	Val
		35				40						45			
Ala	Ala	Ile	Asn	Ser	Gly	Gly	Gly	Ile	Thr	Phe	Tyr	Ala	Asp	Ser	Val
	50					55					60				
Lys	Gly	Arg	Phe	Thr	Ile	Ser	Gln	Asp	Asn	Ala	Lys	Asn	Thr	Val	Tyr
65					70					75					80
Leu	Gln	Met	Asn	Ser	Leu	Lys	Pro	Glu	Asp	Thr	Ala	Ile	Tyr	Tyr	Cys
			85					90						95	
Ala	Ala	Ala	Leu	Ser	Arg	Val	Pro	Gly	Phe	Phe	Pro	Leu	Phe	Pro	Ser
			100					105					110		
Gln	Tyr	Asn	Tyr	Trp	Gly	Gln	Gly	Thr	Gln	Val	Thr	Val	Ser	Ser	
		115					120					125			

<210> 21
 <211> 115
 <212> PRT
 <213> Camelus dromedarius

<400> 21															
Gln	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln	Pro	Gly	Gly
1			5					10						15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe	Ser	Asn	Tyr
			20					25					30		
Trp	Met	Tyr	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu	Glu	Trp	Val
		35				40						45			
Ser	Gln	Ile	Asn	Ser	Gly	Gly	Gly	Thr	Thr	Tyr	Ser	Thr	Asp	Ser	Val
	50					55					60				
Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn	Thr	Leu	Tyr
65					70					75					80
Leu	Gln	Met	Asn	Ser	Leu	Lys	Pro	Glu	Asp	Thr	Ala	Met	Tyr	Tyr	Cys
			85					90						95	

41j

Ala Thr Asp Ser Thr Gly Ser His Arg Gly Gln Gly Thr Gln Val Thr
 100 105 110
 Val Ser Ser
 115

<210> 22
 <211> 124
 <212> PRT
 <213> Camelus dromedarius

<400> 22
 Gln Val Gln Leu Gln Glu Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
 1 5 10 15
 Phe Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Tyr Ser Thr Cys
 20 25 30
 Ser Met Ala Trp Tyr Arg Gln Ala Pro Gly Lys Glu Arg Glu Leu Val
 35 40 45
 Ser Ser Ile Arg Asn Asp Gly Ser Thr Ala Tyr Ala Asp Ser Val Lys
 50 55 60
 Gly Arg Phe Thr Ile Ser Gln Asp Asn Ala Lys Asn Thr Val Tyr Leu
 65 70 75 80
 Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Met Tyr Tyr Cys Asn
 85 90 95
 Ile Arg Ile Gly Val Gly Pro Gly Gly Thr Cys Ser Ile Tyr Ala Pro
 100 105 110
 Tyr Trp Gly Glu Gly Thr Gln Val Thr Val Ser Ser
 115 120

<210> 23
 <211> 127
 <212> PRT
 <213> Camelus dromedarius

<400> 23
 Gln Val Gln Leu Gln Glu Ser Gly Gly Gly Ser Val Gln Ala Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Ile Ser Arg Ser Thr Tyr
 20 25 30
 Phe Met Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val
 35 40 45
 Ala Val Ile Asn Tyr Gly Thr Thr Thr Pro Tyr Tyr Pro Asp Ser Val
 50 55 60
 Lys Gly Arg Phe Thr Val Ser Arg Asp Ser Ser Lys Asn Thr Val Tyr
 65 70 75 80
 Leu Arg Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Ile Tyr Tyr Cys
 85 90 95
 Ala Ala Ala Ser Thr Trp Val Pro Gly Phe Phe Pro Leu Phe Ala Ser
 100 105 110
 Gln Tyr Asn Ser Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser
 115 120 125

<210> 24
 <211> 128
 <212> PRT
 <213> Camelus dromedarius

41k

<400> 24

Gln	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Ser	Val	Gln	Ala	Gly	Gly
1				5					10					15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Ala	Thr	Ala	Ser	Asp	Tyr
			20					25					30		
Cys	Met	Gly	Trp	Phe	Arg	Gln	Ala	Pro	Gly	Lys	Glu	Arg	Glu	Gly	Val
		35					40					45			
Ala	Ala	Ile	Ser	Arg	Gly	Gly	Met	Thr	Tyr	His	Val	Asp	Ser	Val	Arg
	50					55					60				
Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asn	Asn	Ala	Gln	Asn	Thr	Val	Tyr	Leu
65					70					75					80
Gln	Met	Asn	Ser	Leu	Lys	Pro	Glu	Asp	Thr	Ala	Thr	Tyr	Ser	Cys	Ala
				85					90					95	
Ala	Val	Ser	Cys	Ala	Gly	Ala	Trp	Phe	Ala	Asn	Arg	Ala	Leu	Arg	Glu
			100					105					110		
Ser	Ala	Phe	Thr	Tyr	Trp	Gly	Pro	Gly	Thr	Gln	Val	Thr	Val	Ser	Ser
		115					120					125			

<210> 25

<211> 120

<212> PRT

<213> Lama glama

<400> 25

Gln	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln	Ala	Gly	Gly
1				5					10					15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Arg	Ile	Phe	Asp	Leu	Arg
			20					25					30		
Asp	Met	Gly	Trp	Tyr	Arg	Gln	Val	Pro	Gly	Lys	Gln	Arg	Glu	Leu	Val
		35					40					45			
Ala	Ala	Ile	Thr	Ser	Gly	Gly	Thr	Ser	Asn	Tyr	Ala	Asp	Ser	Val	Lys
	50					55					60				
Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn	Thr	Val	Tyr	Leu
65					70					75					80
Gln	Met	Asn	Ser	Leu	Lys	Pro	Glu	Asp	Thr	Ala	Val	Tyr	Tyr	Cys	Asn
				85					90					95	
Ala	Lys	Asn	Phe	Phe	Ser	Ala	Ser	Gly	Tyr	Phe	Leu	Tyr	Trp	Gly	Lys
			100					105					110		
Gly	Thr	Gln	Val	Thr	Val	Ser	Ser								
		115					120								

<210> 26

<211> 121

<212> PRT

<213> Lama glama

<400> 26

Gln	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln	Pro	Gly	Gly
1				5					10					15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe	Glu	Thr	Gln
			20					25					30		
Tyr	Met	Thr	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Pro	Glu	Tyr	Val
		35					40					45			
Ser	Ser	Ile	Asn	Ser	Gly	Gly	Thr	Ile	Lys	Tyr	Tyr	Ala	Asn	Ser	Ser
	50					55					60				
Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn	Thr	Leu
65					70					75					80

411

Tyr	Leu	Gln	Met	Asn	Asn	Leu	Arg	Pro	Glu	Asp	Thr	Ala	Ile	Tyr	Tyr
			85						90					95	
Cys	Gln	Leu	Gly	Gln	Trp	Ala	Gly	Val	Gly	Ala	Ala	Ser	Ser	Arg	Gly
			100					105						110	
Gln	Gly	Thr	Gln	Val	Thr	Val	Ser	Ser							
		115					120								

<210> 27
 <211> 122
 <212> PRT
 <213> Lama glama

Gln	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln	Pro	Gly	Gly
1			5					10						15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe	Ser	Thr	Tyr
			20					25					30		
Trp	Met	Tyr	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu	Glu	Trp	Val
		35				40						45			
Ser	Ala	Ile	Ser	Thr	Glu	Gly	Gly	Ser	Thr	Arg	Tyr	Ala	Gly	Ser	Val
	50					55				60					
Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Ser	Thr	Leu	Tyr
65					70				75					80	
Leu	Gln	Met	Asp	Ser	Leu	Lys	Ser	Glu	Asp	Thr	Ala	Val	Tyr	Tyr	Cys
			85					90						95	
Ser	Lys	Gly	Thr	Gly	Pro	Phe	Thr	Asp	Ile	Arg	Ser	Thr	Gly	Ser	Arg
			100					105					110		
Gly	Lys	Gly	Thr	Gln	Val	Thr	Val	Ser	Ser						
		115					120								

<210> 28
 <211> 122
 <212> PRT
 <213> Lama glama

Gln	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln	Pro	Gly	Gly
1			5					10						15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe	Ser	Ser	Tyr
			20					25					30		
Trp	Met	Tyr	Trp	Phe	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu	Glu	Arg	Val
		35				40						45			
Ser	Ala	Ile	Asn	Phe	Gly	Gly	Asp	Val	Thr	Tyr	Tyr	Thr	Asp	Ser	Val
	50				55					60					
Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn	Thr	Leu	Tyr
65					70				75					80	
Leu	Gln	Met	Asn	Ser	Leu	Lys	Ser	Glu	Asp	Thr	Ala	Val	Tyr	Tyr	Cys
			85					90						95	
Thr	Lys	Gly	Leu	Ser	Pro	Tyr	Arg	Asp	Leu	Glu	Ser	Ser	Gly	Ser	Arg
			100					105					110		
Gly	Gln	Gly	Thr	Gln	Val	Thr	Val	Ser	Ser						
		115					120								

<210> 29
 <211> 10
 <212> PRT
 <213> Camelus dromedarius

41m

<400> 29

Glu Tyr Thr Tyr Gly Tyr Cys Ser Met Gly
 1 5 10

<210> 30

<211> 16

<212> PRT

<213> Camelus dromedarius

<400> 30

Thr Ile Thr Ser Asp Gly Ser Thr Ser Tyr Val Asp Ser Val Lys Gly
 1 5 10 15

<210> 31

<211> 13

<212> PRT

<213> Camelus dromedarius

<400> 31

Lys Thr Cys Ala Asn Lys Leu Gly Ala Lys Phe Ile Ser
 1 5 10

<210> 32

<211> 10

<212> PRT

<213> Camelus dromedarius

<400> 32

Gly Tyr Thr Tyr Ser Thr Cys Ser Met Ala
 1 5 10

<210> 33

<211> 16

<212> PRT

<213> Camelus dromedarius

<400> 33

Ser Ile Arg Asn Asp Gly Ser Thr Ala Tyr Ala Asp Ser Val Lys Gly
 1 5 10 15

<210> 34

<211> 16

<212> PRT

<213> Camelus dromedarius

<400> 34

Arg Ile Gly Val Gly Pro Gly Gly Thr Cys Ser Ile Tyr Ala Pro Tyr
 1 5 10 15

<210> 35

<211> 10

<212> PRT

<213> Lama glama

41n

<400> 35

Gly	Phe	Thr	Phe	Glu	Thr	Gln	Tyr	Met	Thr
1				5					10

<210> 36

<211> 18

<212> PRT

<213> Lama glama

<400> 36

Ser	Ile	Asn	Ser	Gly	Gly	Thr	Ile	Lys	Tyr	Tyr	Ala	Asn	Ser	Ser	Val
1				5					10					15	

Lys Gly

<210> 37

<211> 11

<212> PRT

<213> Lama glama

<400> 37

Gly	Gln	Trp	Ala	Gly	Val	Gly	Ala	Ala	Ser	Ser
1				5					10	

<210> 38

<211> 115

<212> PRT

<213> Camelus dromedarius

<400> 38

Asp	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln	Pro	Gly	Gly
1				5					10					15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Glu	Phe	Thr	Phe	Gly	Ser	Tyr
			20					25					30		
Trp	Met	Tyr	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu	Glu	Trp	Val
		35					40					45			
Ala	Gln	Ile	Asn	Ala	Arg	Gly	Ser	Thr	Ile	Tyr	Tyr	Val	Asp	Ser	Val
	50					55					60				
Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn	Thr	Leu	Tyr
65					70					75					80
Leu	Gln	Met	Asn	Ser	Leu	Lys	Pro	Glu	Asp	Thr	Ala	Val	Tyr	Tyr	Cys
			85						90					95	
Ala	Thr	Asp	Ser	Arg	Gly	Thr	His	Lys	Gly	Gln	Gly	Thr	Gln	Val	Thr
			100					105					110		
Val	Ser	Ser													
															115

<210> 39

<211> 115

<212> PRT

<213> Camelus dromedarius

<400> 39

Asp	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Leu	Val	Arg	Pro	Gly	Gly
1				5					10					15	
Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe	Ala	Asn	Tyr
			20					25					30		

410

```

Trp Leu Tyr Trp Val Arg Asp Ala Pro Gly Lys Gly Ile Glu Trp Val
      35              40              45
Ser Gln Ile Gly Pro Ser Gly Arg Ser Thr Tyr Tyr Ala Asp Ala Val
      50              55              60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Lys Thr Leu Tyr
65              70              75              80
Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Ser Ala Val Tyr Tyr Cys
      85              90              95
Ala Thr Ser Ser Gly Gly Asn Glu Arg Gly Gln Gly Thr Gln Val Thr
      100              105              110
Val Ser Ser
      115

```

```

<210> 40
<211> 115
<212> PRT
<213> Camelus dromedarius

```

```

<400> 40
Asp Val Gln Leu Gln Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1              5              10              15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Ala Phe Ser Ser Tyr
      20              25              30
Trp Met Tyr Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
      35              40              45
Ser Gln Val Asn Ser Asp Gly Gly Ser Thr Tyr Tyr Val Asp Ser Val
      50              55              60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65              70              75              80
Leu His Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys
      85              90              95
Ala Thr Asp Ser Ser Gly Arg Tyr Arg Gly Gln Gly Thr Gln Val Thr
      100              105              110
Val Ser Ser
      115

```

```

<210> 41
<211> 115
<212> PRT
<213> Camelus dromedarius

```

```

<400> 41
Asp Val Gln Leu Gln Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1              5              10              15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
      20              25              30
Trp Met Tyr Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
      35              40              45
Ser Gln Ile Asn Ser Ser Gly Gly Thr Thr Tyr Tyr Ala Asp Ser Val
      50              55              60
Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
65              70              75              80
Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys
      85              90              95
Ala Thr Gly Ser Ala Gly Gln Gly Lys Gly Gln Gly Thr Gln Val Thr
      100              105              110
Val Ser Ser
      115

```

41p

<210> 42
 <211> 115
 <212> PRT
 <213> Camelus dromedarius

<400> 42
 Asp Val Gln Leu Gln Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn Tyr
 20 25 30
 Trp Met Tyr Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
 35 40 45
 Ser Gln Ile Asp Gly Gly Gly Arg Lys Thr Tyr Tyr Ala Asp Ser Leu
 50 55 60
 Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr
 65 70 75 80
 Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Met Tyr Tyr Cys
 85 90 95
 Ala Thr Asp Ser Ala Gly Ser His Arg Gly Gln Gly Thr Gln Val Thr
 100 105 110
 Val Ser Ser
 115

<210> 43
 <211> 115
 <212> PRT
 <213> Camelus dromedarius

<400> 43
 Asp Val Gln Leu Gln Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
 1 5 10 15
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Pro Phe Ser Val Tyr
 20 25 30
 Trp Met Tyr Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
 35 40 45
 Ser Gln Ile Asp Ser Gly Gly Tyr Thr Thr Tyr Tyr Thr Asp Ser Val
 50 55 60
 Lys Gly Arg Phe Ser Ala Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
 65 70 75 80
 Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Thr Asp Ser Ile Gly Ser Asn Lys Gly Gln Gly Thr Gln Val Thr
 100 105 110
 Val Ser Ser
 115

<210> 44
 <211> 23
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Primer CALL001

<400> 44
 gtcctggctg ctcttctaca agg

41q

<210> 45
 <211> 23
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Primer CALL002

<400> 45
 ggtacgtgct gttgaactgt tcc

23

<210> 46
 <211> 29
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> AE6

<400> 46
 gatgtgcagc tgcaggagtc tggaggagg

29

<210> 47
 <211> 35
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Primer FR4FOR

<400> 47
 ggactagtgc ggccgctgca gacggtgacc tgggt

35

<210> 48
 <211> 15
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> FRET peptide substrate

<400> 48
 Ser Glu Val Asn Leu Asp Ala Glu Phe Arg Lys Arg Arg Arg Arg
 1 5 10 15

29775-103

42

CLAIMS:

1. A single domain antibody, devoid of a light chain, specifically binding to beta-site APP-cleaving enzyme 1 (BACE1), which inhibits the activity of BACE1 as determined in a cellular assay.
- 5 2. The single domain antibody according to claim 1 which is derived from camelids.
3. The single domain antibody according to claim 1 or 2 which comprises the amino acid sequence as set forth in SEQ ID NO: 6, 22, or 26.
4. The single domain antibody according to any one of claims 1 to 3 for
10 use in treatment of Alzheimer's disease.
5. A pharmaceutical composition comprising the single domain antibody according to any one of claims 1 to 3 and at least one pharmaceutically acceptable carrier.
6. Use of the single domain antibody according to any one of claims 1 to 3
15 or the pharmaceutical composition according to claim 5 for the manufacture of a medicament to treat Alzheimer's disease.
7. Use of a single domain antibody comprising the amino acid sequence as set forth in SEQ ID NO: 6, 22 or 26 for the manufacture of a medicament to treat Alzheimer's disease.

	< -- Framework-1 -- >	< - CDR1 - >	< Framework-2 >	< --- CDR2 --- >
	5 10 15 20 25 30 35 40 45 50 a b 55 60 65			
Nb_B1	DVQLQESGGGSVQAGGSLRLSCVAS	GWTYSSNSLSMA	WFRQAPGKEREGVA	--TI-TSYVGRTYYADSVKG
NB_B2	DVQLQESGGGSVQAGGSLRLSCAVS	GFSYS--PYIMG	WFRQAPGKEREGVA	--AI-RKGIGTTYADSVKG
Nb_B3	DVQLQESGGGSVQAGGSLRLSCAVS	GYTYN--IYTMA	WFRQAPGKEREGVA	--GIY-SPGGTTYADSVKG
Nb_B5	DVQLQESGGGSVQAGGSLRLSCAAS	GYTFT--KYPMG	WFRQAPGKE <u>C</u> ELVS	--SI-IS-GGVTTYASSVKG
Nb_B8	DVQLQESGGGSVQAGGSLRLSCARS	GGTVS--IPYMA	WFRQGPGKEREGVA	--AIYD-GRAKT-YAGSLQG
Nb_B9	DVQLQESGGGSVQAGGSLRLSCAAS	EYTYG--Y <u>C</u> SMG	WYRQAPGKERELVS	--TI-TSDGS-TSYVDSVKG
Nb_B10	DVQLQESGGGSVQPGGSLRLSCAAS	GYFYS--RWYMG	WFRQAPGKEREGVA	--AI-NSGGSITSYADSVKG
Nb_B11	DVQLQESGGGSAQAGGSLRLSCEVS	GYTYS--GYFMG	WFRQAPGEEREGVA	--AI-DTNGGRTWYADSVKG
Nb_B12	DVQLQESGGGSVQAGGSLRLSCVAS	GFTYR--RYFMG	WFRQAPGKEREAVA	--TM-FS <u>C</u> GGTTYADSVKG
Nb_B15	DVQLQESGGGSVQAGGSLRLSCAAS	GYSYS---YYIG	WFRQAPGKEREGVA	AIAIVNSGGGRITYYADSVKG
Nb_B16	DVQLQESGGGSVQAEGSLRLSCTAS	GYTYS----LMG	WFRQAPGKEREGVA	--VI-NSGVGTTYADSVKG
Nb_B21	DVQLQESGGGSVQAGGSLRLSCAAS	GYTSE--MNRFA	WLRQAPGKDREVVA	--VIFPTARGAKFYSDSVNG
Nb_B25	QVQLQESGGGTVQAGGSLRLSCAAS	GYTYR--SY <u>C</u> MG	WFRQAPGKEREEVA	--SI-NSDQGSTRYAASVKG
Nb_B26	DVQLQESGGGSVQAGGSLRLSCAAS	GYAFS--SYIMG	WFRQAPGREREEVT	--GI-TQIGGTTYADSVKG
Nb_B4	DVQLQESGGGLVQPGGSLRLSCAAS	EFTFG--SYWMY	WVRQAPGK <u>GLEW</u> VA	--QI-NARGSTIYYVDSVKG
Nb_B6	DVQLQESGGGLVVRPGGSLRLSCAAS	GFTFA--NYWLY	WVRDAPGK <u>GIEW</u> VS	--QI-GPSGRSTYYADAVKG
Nb_B7	DVQLQESGGGLVQPGGSLRLSCAAS	GFAFS--SYWMY	WVRQAPGK <u>GLEW</u> VS	--QV-NSDGGSTYYVDSVKG
Nb_B13	DVQLQESGGGLVQPGGSLRLSCAAS	GFTFS--SYWMY	WVRQAPGK <u>GLEW</u> VS	--QI-NSSGGTTYADSVKG
Nb_B14	DVQLQESGGGLVQPGGSLRLSCAAS	GFTFS--NYWMY	WVRQAPGK <u>GLEW</u> VS	--QI-DGGGRKTTYADSLKG
Nb_B24	DVQLQESGGGLVQPGGSLRLSCAAS	GFPFS--VYWMY	WVRQAPGK <u>GLEW</u> VS	--QI-DSGGYTTYTTDSVKG

Figure 1 continued

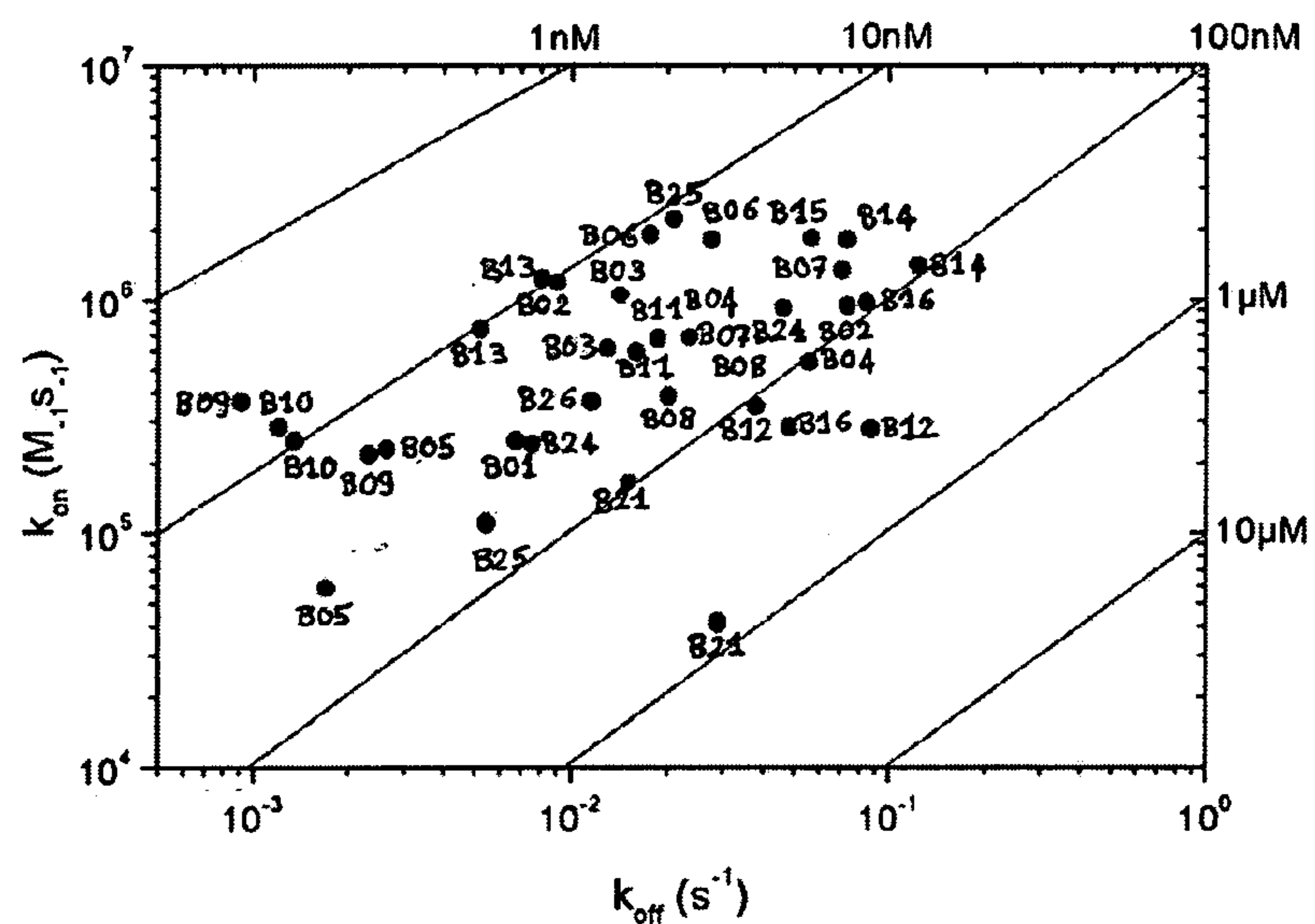
< ----- Framework-3 ----- >	< ----- CDR3 ----- >	Framework-4	
70 75 80 abc 85 90		105 110	
RFTISRDHAKST--VYLQIDSLKPEDTATYYCAA	EYLGGs-FLSTGA-----YKY	WGQGTQVTVSS	Nb_B1
RFTFSQDDAKNT--MYLQMNSLKPEDTAIFYCAV	GHYRAYATTsFDPRR-----YDY	WGQGTQVTVSS	Nb_B2
RFTISQDNAKNT--VYLQMNSLAPEDTAIYYCAA	RGGLLSRVLKEAG-----YNA	WGQGTQVTVSS	Nb_B3
RFTISRDNakNT--VYLQMNSLKPEDTAVYYCAA	QYPYSSsWPR---CPFR---IGY	WGQGTQVTVSS	Nb_B5
RFTISQDNDKNT--LYLQMNSLKPDDTAVYYCAA	GNGGGNWL---RPSE-----YNY	WGKGTQVTVSS	Nb_B8
RFTISQDNAKNT--VYLQMNSLKPEDTAIYYCYT	KTCA--NK-LGAK-----FIS	WGQGTQVTVSS	Nb_B9
RFTISQDNAKNT--VYLQMNSLKPEDTAIYYCAA	ALSRVPGFFP---LFPSQ---YNY	WGQGTQVTVSS	Nb_B10
RFTISHDNAEST--VYLQMNSLQPEDTAIFYCAA	RRPPGGsWYPPP---LRKYSYNF	WGQGTQVTVSS	Nb_B11
RFTATQDNAKNT--VYLQMNNLKPEDTAIYYCAA	ASGCWYDGSPAA----RSVDVSF	WGHGTQVTVSS	Nb_B12
RFTISQGNdkNT--VYLQMNSLKPEDTAIYYCAA	RSLSWYSHPL---LQPSQ---FNN	WGQGTQVTVSS	Nb_B15
RFTISQDNAKST--VYLQMNSLKPEDTAIYYCAA	RRSWFTGMTTTQALDPDW---FSY	WGQGTQVTVSS	Nb_B16
RFTISQDTAKNT--VYLQMNSLEPEDTAMYFCAA	SANAMTGFQPSG-----YTY	WGQGTQVTVSS	Nb_B21
RFTSSQDNANNTVTVYLMNSLKPEDTAIYYCAA	NDGCAYRVYRGGAYG-----YNF	WGQGTQVTVSS	Nb_B25
RFTISRDNakNT--VYLQMDSLKPEDTAIYYCAK	LRRPFYYPLLERPSEGD---FDY	WGQGTQVTVSS	Nb_B26
RFTISRDNakNT--LYLQMNSLKPEDTAVYYCAT	DSRGTH-----	KGQGTQVTVSS	Nb_B4
RFTISRDNakKT--LYLQMNSLKPEDSAVYYCAT	SSGGNE-----	RGQGTQVTVSS	Nb_B6
RFTISRDNakNT--LYLHMNSLKPEDTAVYYCAT	DSSGRY-----	RGQGTQVTVSS	Nb_B7
RFTISRDNakNT--LYLQMNSLKPEDTAVYYCAT	GSAGQG-----	KGQGTQVTVSS	Nb_B13
RFTISRDNakNT--LYLQMNSLKPEDTAMYCAT	DSAGSH-----	RGQGTQVTVSS	Nb_B14
RFSASRDNSKNT--LYLQMNSLKPEDTAVYYCAT	DSIGSN-----	KGQGTQVTVSS	Nb_B24

BDS/NAN/V281

3/9

Figure 2

A



B

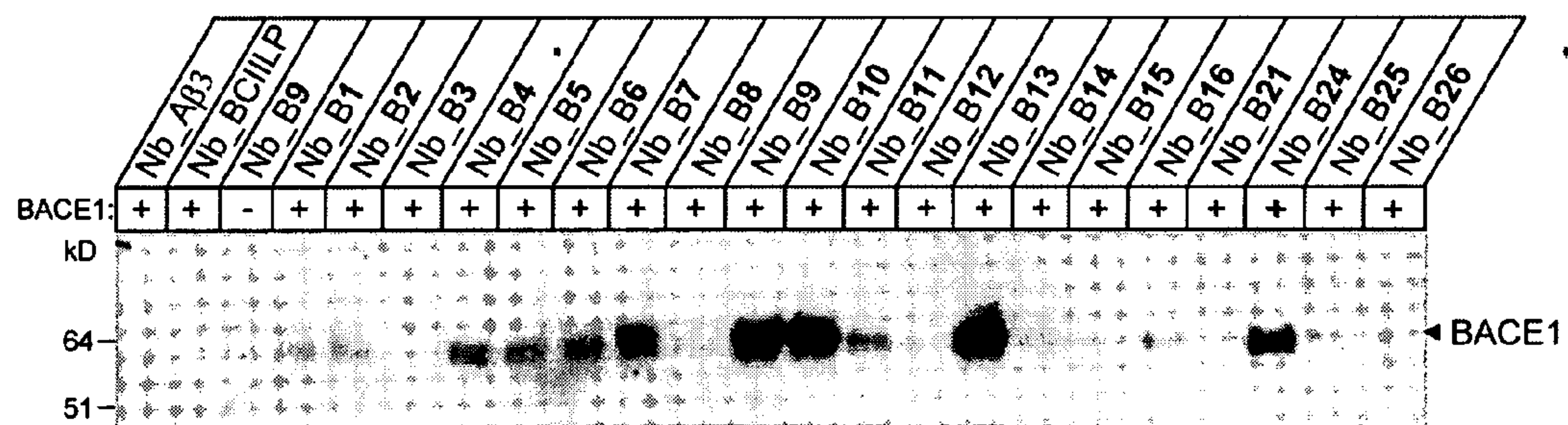


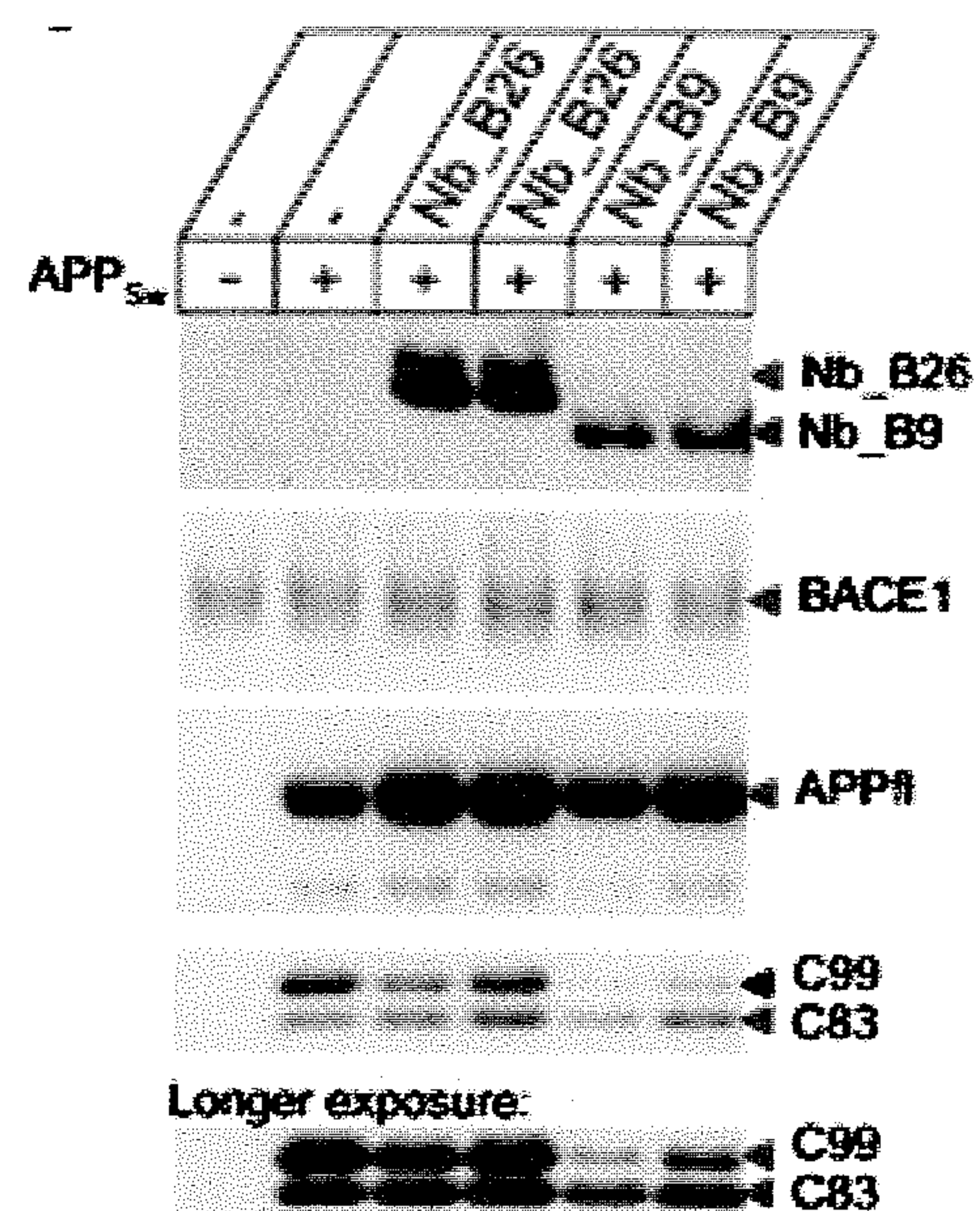
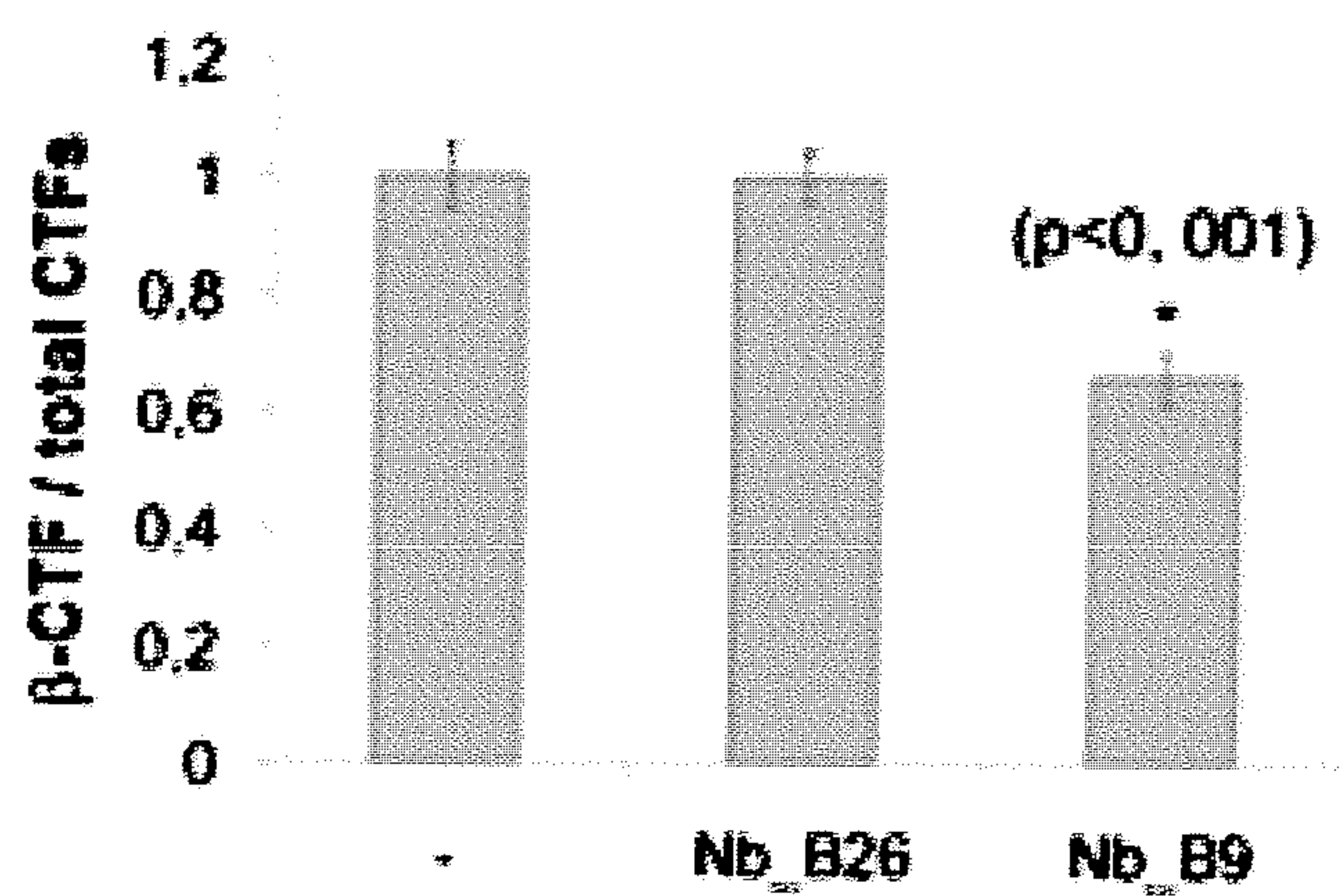
Figure 3**A****B****C**

Figure 4

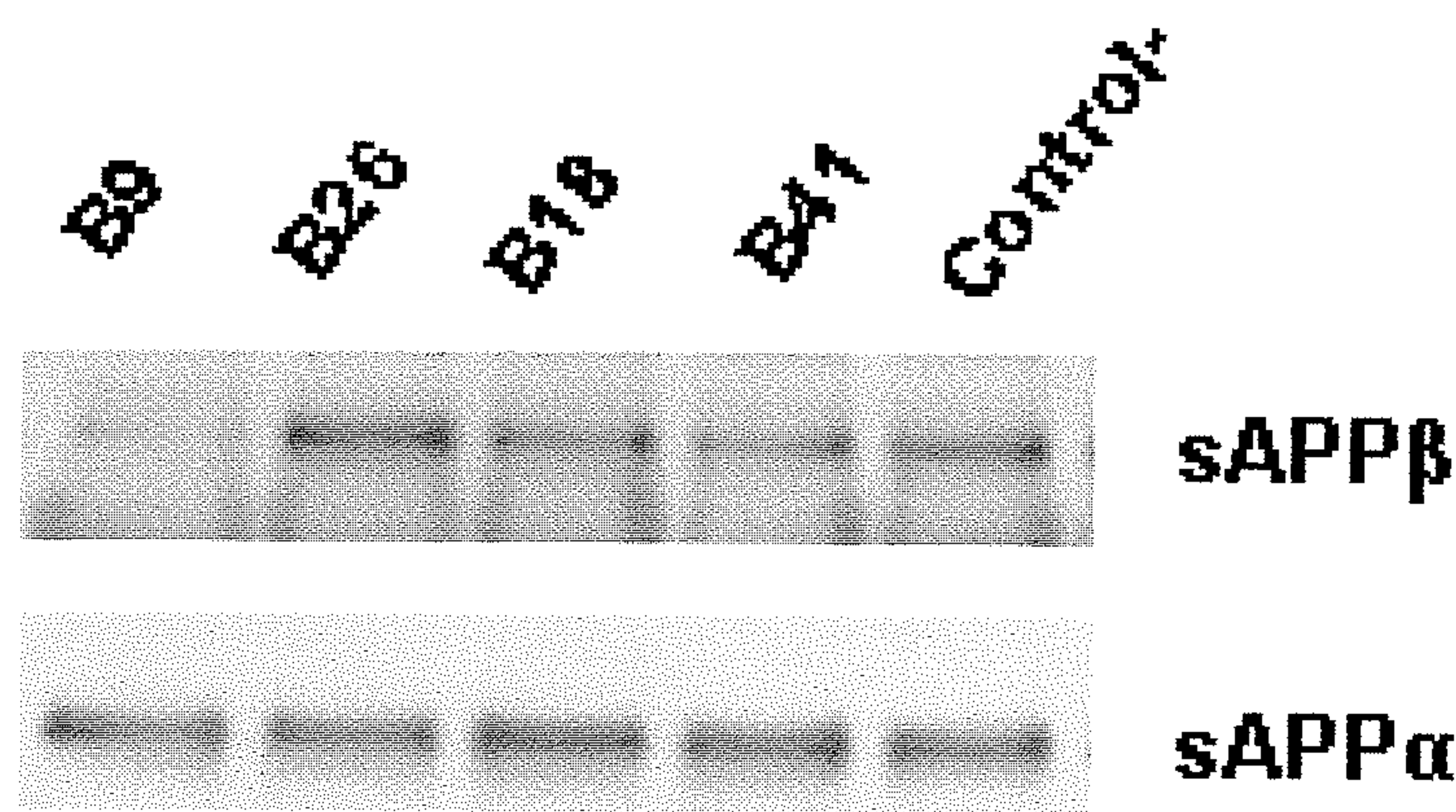


Figure 5

< -----Framework1----- > < -CDR1- > < Framework2 > < -----CDR2----->			
Nb_B9	DVQLQESGGGSVQAGGSLRLS	CAAS EYTYGYC-SMG WYRQAPGKERELVS	TITSDGS-TS-YVDSVKG
Nb_1B3	QVQLQESGGGSVQAGGSLRLS	CAAS EYTDSTY-YMA WFRQAPGKERGGVA	TLASRYD-TTYYADSVKG
Nb_10C2	QVQLQESGGGSVQAGGSLRLS	CAAS RDILTLY-YMG WFRQAPGKEREGVA	AISSDII-FTSYANSVKG
Nb_12B6	QVQLQESGGGSVQAGGSLRLS	CAAS RDILTLY-YMG WFRQAPGKEREGVA	AISSDII-FTSYANSVKG
Nb_10B5	QVQLQESGGGSVQAGGSLRLS	CAHS ---NTYPTYMG WFRQAPGKEREGVA	AIYTG DG-TTYYGDSVKG
Nb_13A5	QVQLQESGGGSVQAGGSLRLS	CAHS ---NTYPTYMG WFRQAPGKEREGVA	AIYTG DG-TTYYGDSVKG
Nb_2C6	QVQLQESGGGSVQAGGSLRLS	CAAS GFTSSVY-YIS WFRQAPGKEREGVA	AINSGGG-ITFYADSVKG
Nb_6A4	QVQLQESGGGLVQPGGSLRLS	CAAS GFTFSNY-WMY WVRQAPGKGLEWVS	QINSGGG-TTYSTDSVKG
Nb_10C4	QVQLQESGGGSVQAGGFLRLS	CAAS GYTYSTC-SMA WYRQAPGKERELVS	SI--RNDGSTAYADSVKG
Nb_13B6	QVQLQESGGGSVQAGGSLRLS	CAAS GISRSTY-FMG WFRQAPGKEREGVA	VINYGTT-TPYYPDSVKG
Nb_1A4	QVQLQESGGGSVQAGGSLRLS	CAAS GATASDY-CMG WFRQAPGKEREGVA	AI-SRGG-MTYHVD SVRG
Nb_2B6	QVQLQESGGGLVQAGGSLRLS	CAAS GRIF-DLRDMG WYRQVPGKQRELVA	AI-TSGG-TSNYADSVKG
Nb_4A2	QVQLQESGGGLVQPGGSLRLS	CAAS GFTFETQ-YMT WVRQAPGKGPEYVS	SINSGGTIKYYANSSVKG
Nb_1D4	QVQLQESGGGLVQPGGSLRLS	CAAS GFTFSTY-WMY WVRQAPGKGLEWVS	AISTEGGS-TRYAGSVKG
Nb_9D3	QVQLQESGGGLVQPGGSLRLS	CAAS GFTFSSY-WMY WFRQAPGKGLERVS	AINFGGDV-TYYTDSVKG
< -----Framework2----- > < -----CDR3----- > < Framework3>			
RFTISQDNAKNTVY LQMNSLKPEDTAKYYCYT	KTCANKLGAKFI-----S	WGQGTQVTVSS	Nb_B9
RFTISQDRAKNTVY LQMNSLKPEDTGIYYCAA	SPRR-PGFFPLDPSQYNY	WGQGTQVTVSS	Nb_1B3
RFTISRDKDKNTVY LQMNSLKPEDTAIYYCAA	ASTWVPGFFPLFASQYNS	WGQGTQVTVSS	Nb_10C2
RFTISRDEDKNTVY LQMNSLKPEDTAIYYCAA	ASTWVPGFFPLFASQYNS	WGQGTQVTVSS	Nb_12B6
RFTISQDNAKNTVY LQMNSLKPEDTAIYYCAA	ALSRVPGFFPLFPSQYNY	WGQGTQVTVSS	Nb_10B5
RFTISQDNAKNTVY LQMNSLKPEDTAIYYCAA	ALSRVPGFFPLFPSQYNY	WGQGTQVTVSS	Nb_13A5
RFTISQDNAKNTVY LQMNSLKPEDTAIYYCAA	ALSRVPGFFPLFPSQYNY	WGQGTQVTVSS	Nb_2C6
RFTISRDNAKNTLY LQMNSLKPEDTAMYYCAT	D-----STGSH	RGQGTQVTVSS	Nb_6A4
RFTISQDNAKNTVY LQMNSLKPEDTAMYYCNI	R-IGVGP-GGTCSIYAPY	WEGGTQVTVSS	Nb_10C4
RFTVSRDSSKNTVY LRMNSLKPEDTAIYYCAA	ASTWVPGFFPLFASQYNS	WGQGTQVTVSS	Nb_13B6
RFTISRNNAQNTVY LQMNSLKPEDTATYSCAA	VSCAGAWFANRALRESAFTY	WGPQTQVTVSS	Nb_1A4
RFTISRDNAKNTVY LQMNSLKPEDTAVYYCNA	KNFFSASGYFL-----Y	WGKGTQVTVSS	Nb_2B6
RFTISRDNAKNTLY LQMNNLRPEDTAIYYCQL	GQ-WAGVGAAS-----S	RGQGTQVTVSS	Nb_4A2
RFTISRDNAKSTLY LQMDSLKSEDTAVYYCSK	GTGPFTDIRSTG-----S	RGKGTQVTVSS	Nb_1D4
RFTISRDNAKNTLY LQMNSLKSEDTAVYYCTK	GLSPYRDLESSG-----S	RGQGTQVTVSS	Nb_9D3

Figure 6

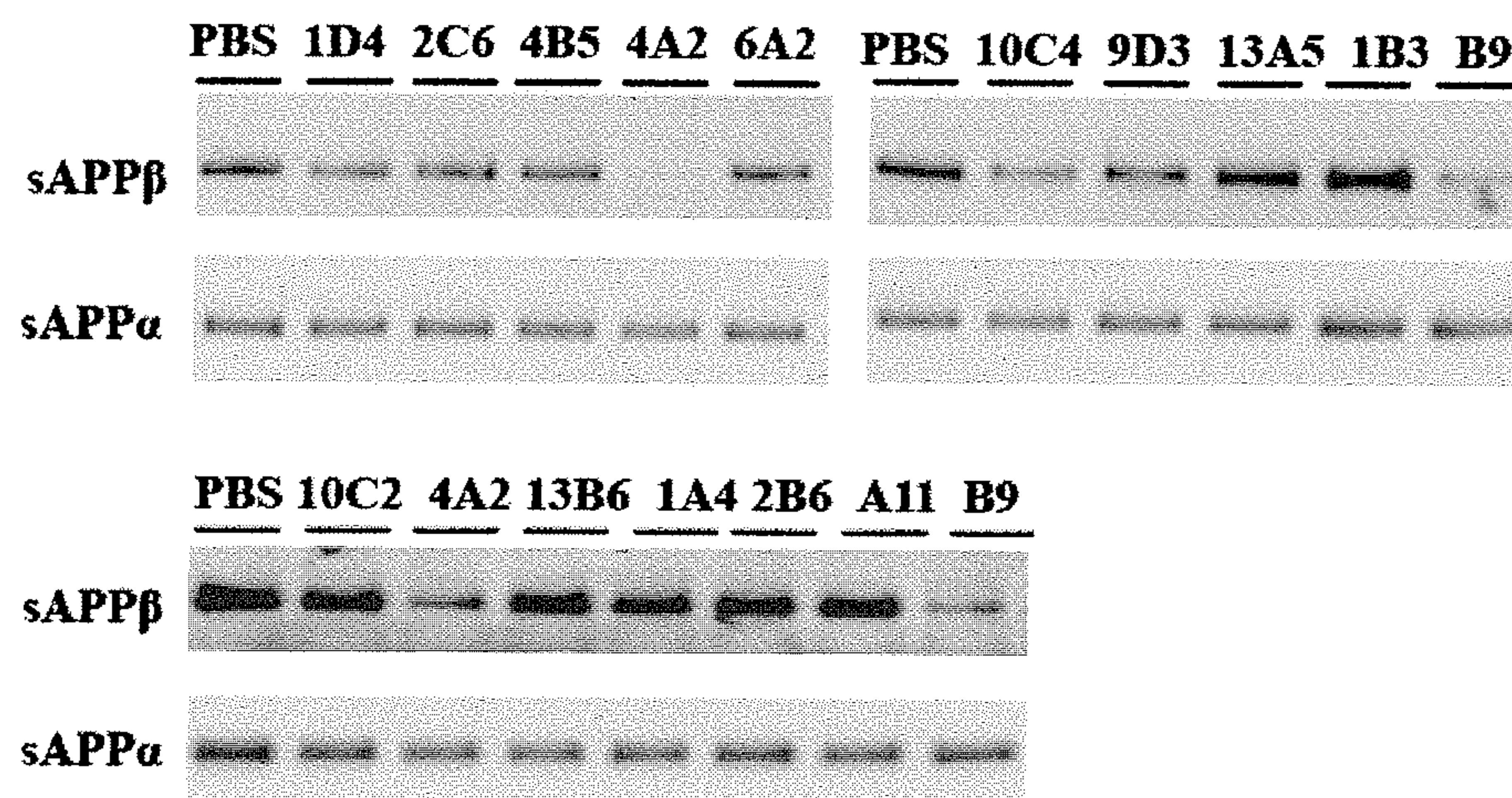


Figure 7

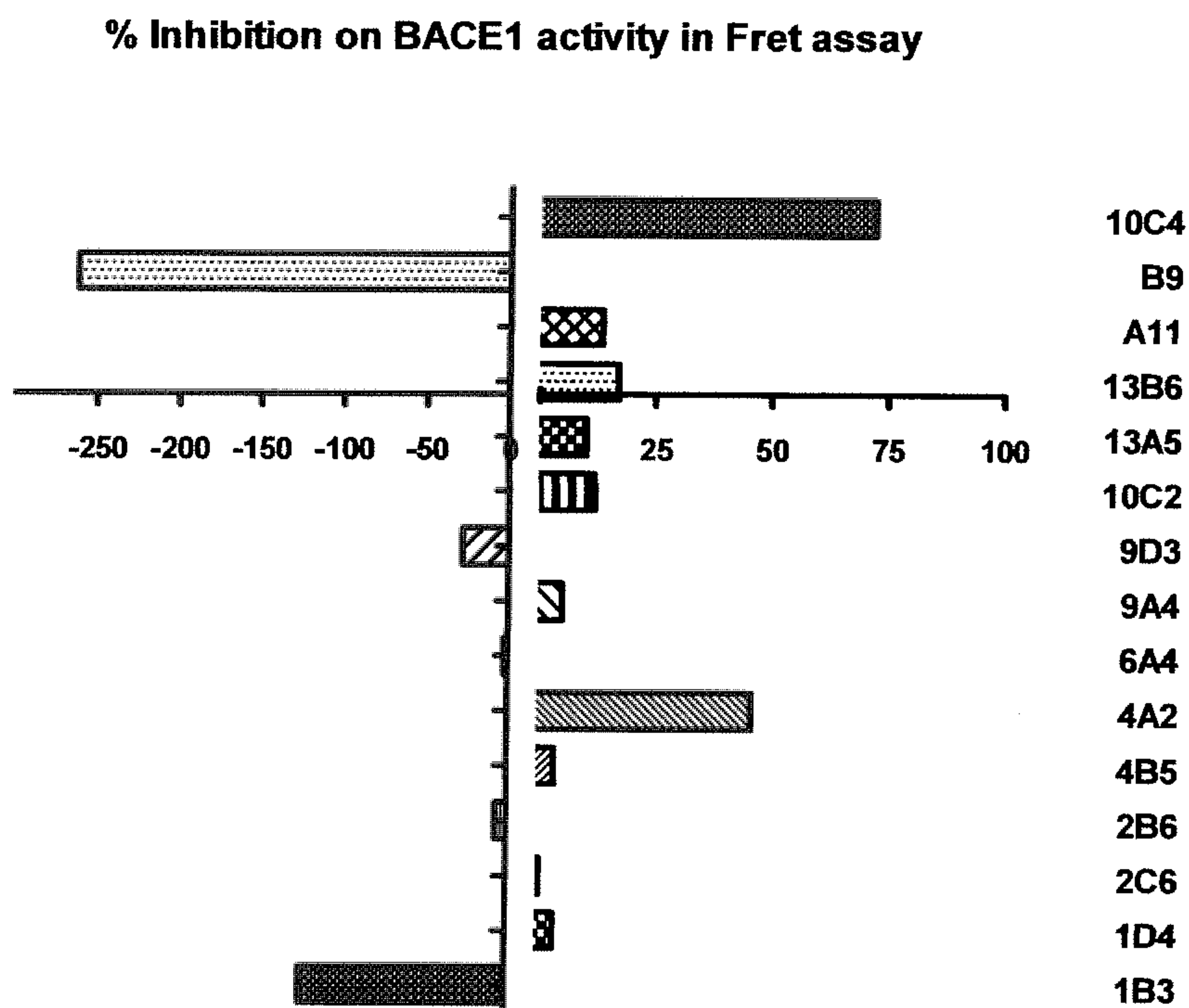
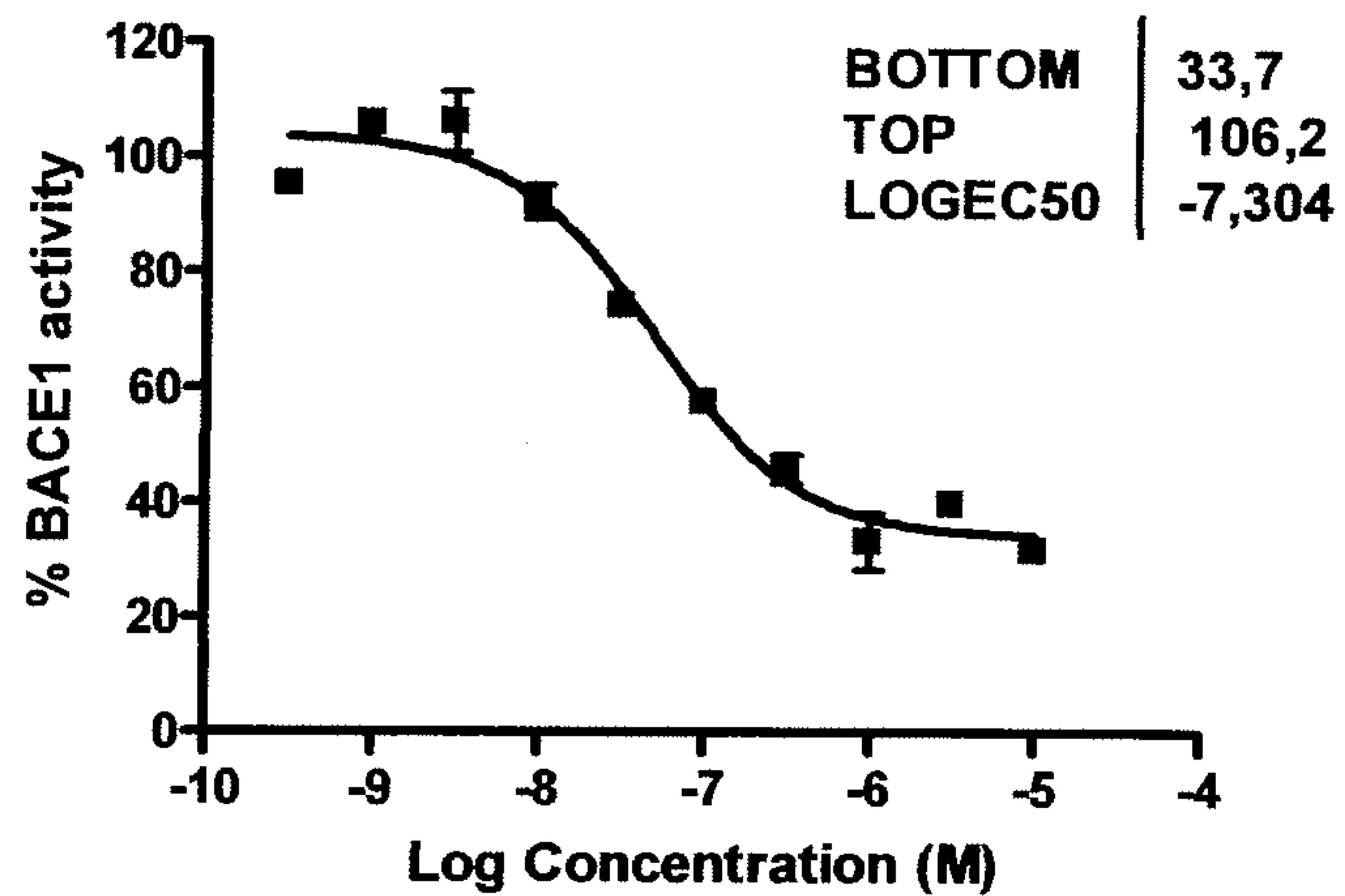
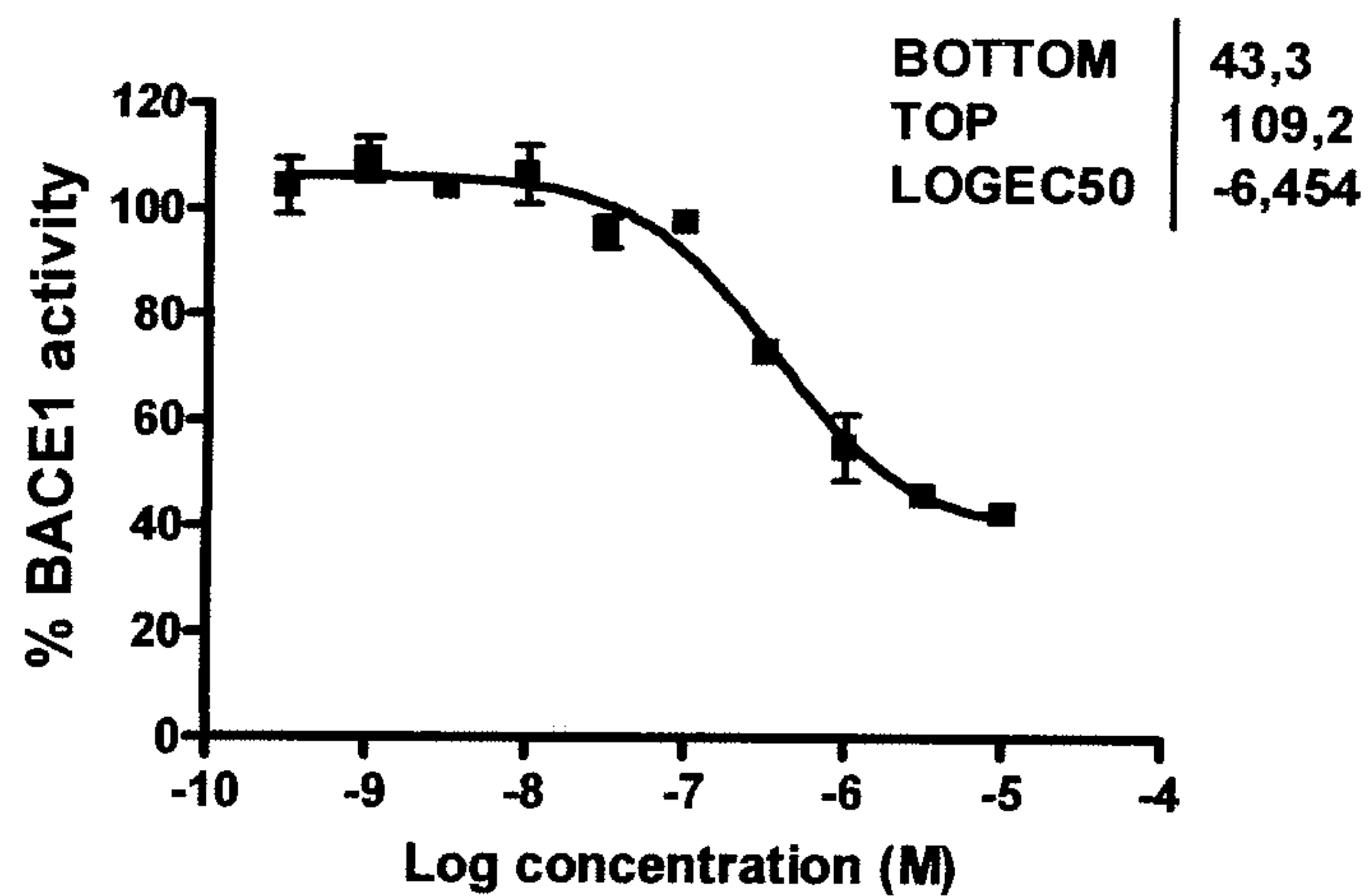


Figure 8

A

10C4 effects on BACE1 in Fret assay

B

4A5 effects on BACE1 in Fret assay

C

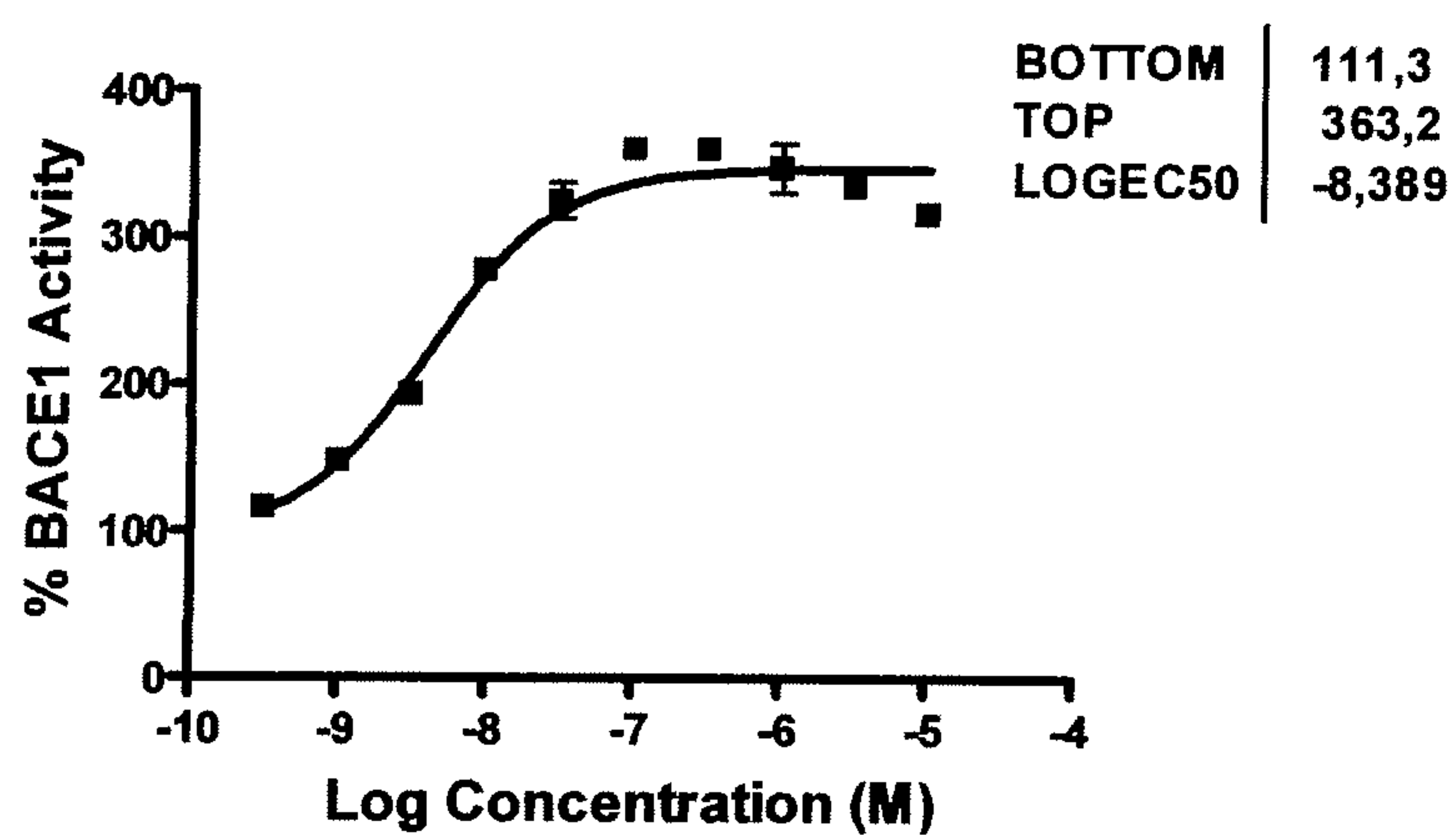
B9 effects on BACE1 in Fret assay

Figure 9

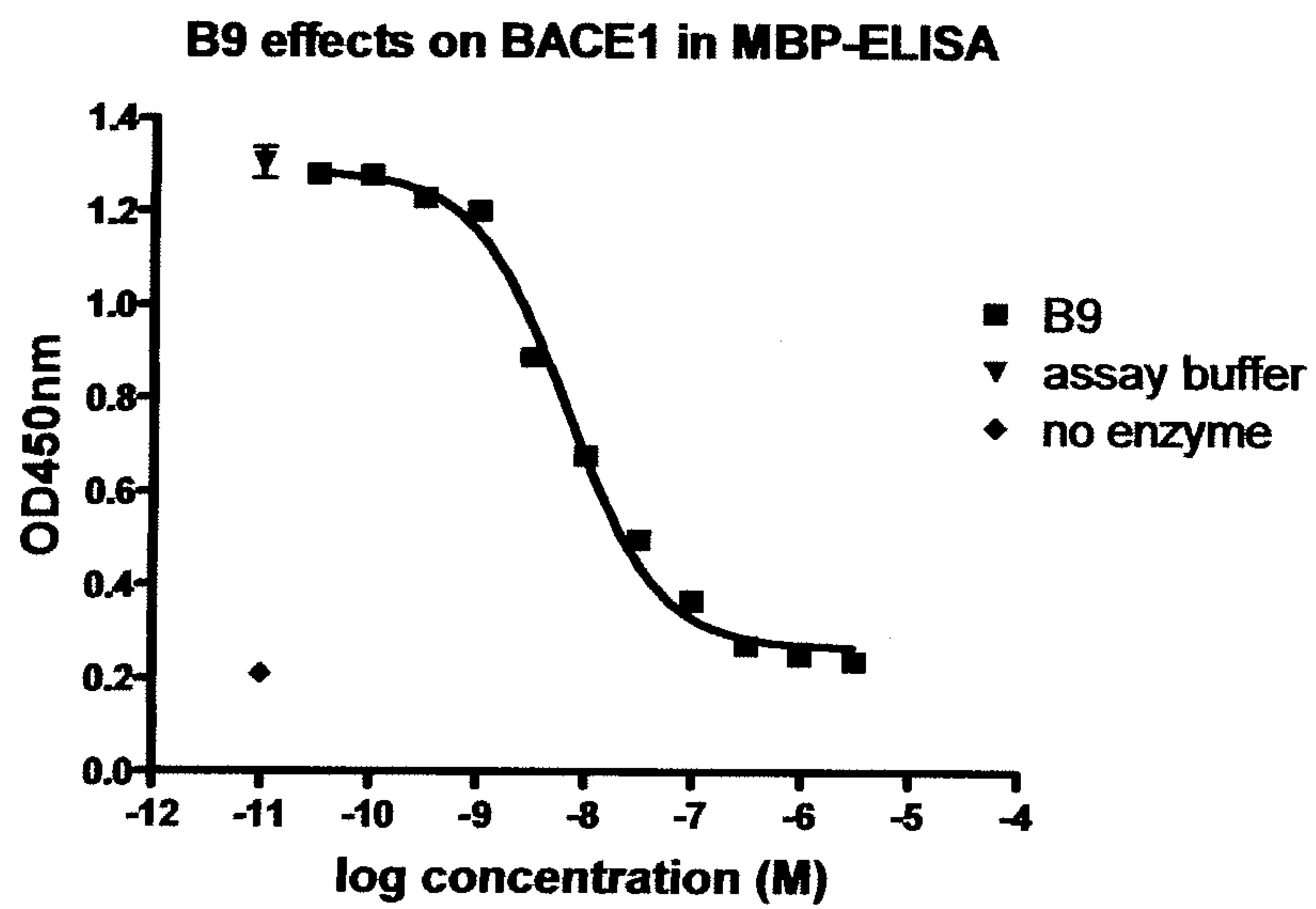


Figure 10

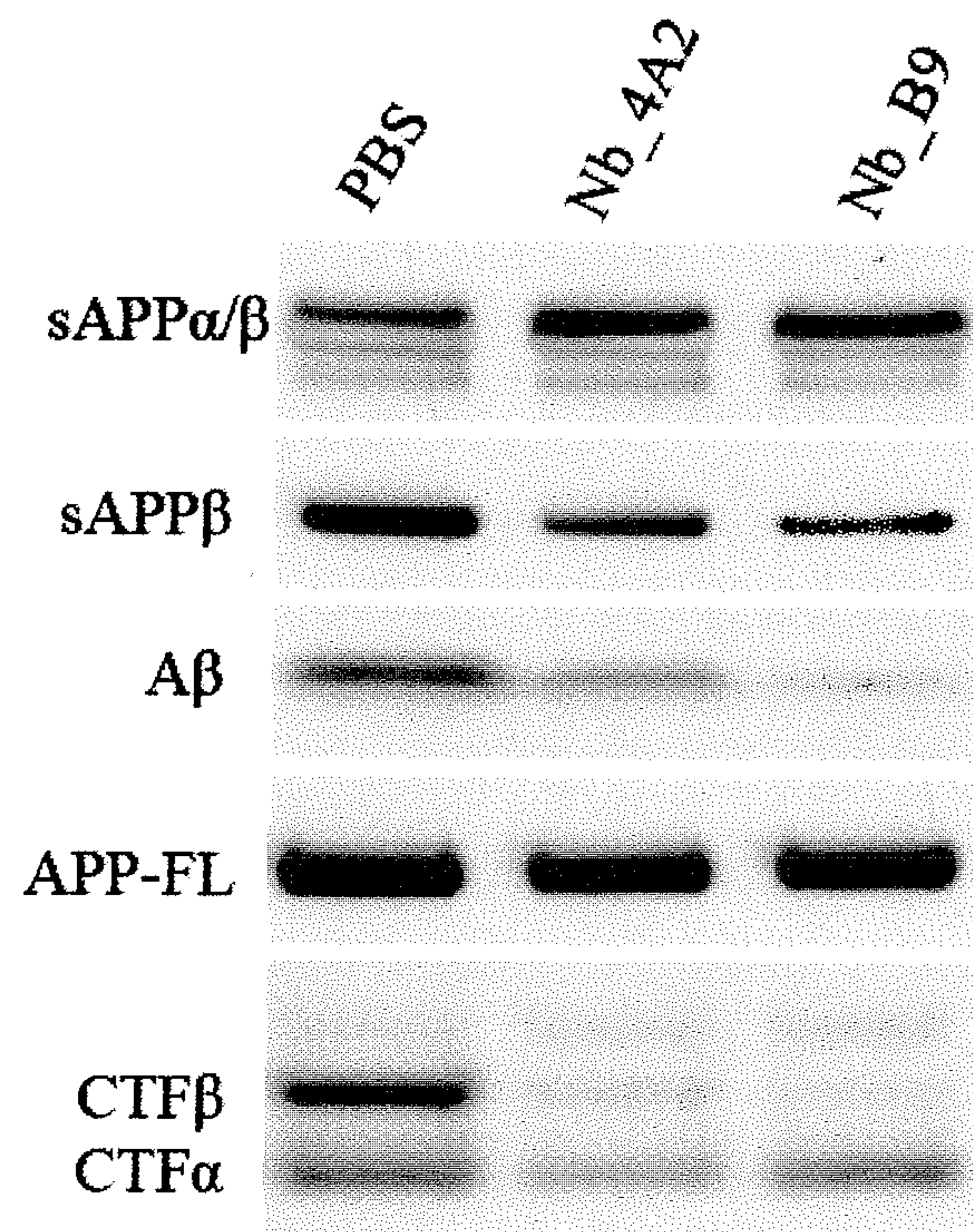


Figure 11

