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RESEARCH ARTICLE

Pharmacological applications of a novel neoepitope antibody to a modified amyloid precursor protein-derived beta-secretase product

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ABSTRACT

We have previously described a novel artificial NFEV β-secretase (BACE1) cleavage site, which when introduced into the amyloid-β precursor protein (APP), significantly enhances APP cleavage by BACE1 in *in vitro* and cellular assays. In this study, we describe the identification and characterization of a single chain fragment of variable region (scFv), specific to the EV neo-epitope derived from BACE1 cleavage of the NFEV-containing peptide, and its conversion to IgG1. Both the scFv displayed on phage and EV-IgG1 show exquisite specificity for binding to the EV neoepitope without cross-reactivity to other NFEV containing peptides or WT-APP KMDA cleavage products. EV-IgG1 can detect as little as 0.3 nmol/L of the EV peptide. EV-IgG1 antibody was purified, conjugated with alkaline phosphatase and utilized in various biological assays. In the BACE1 enzymatic assay using NFEV substrate, a BACE1 inhibitor MRK-3 inhibited cleavage with an IC_{50} of 2.4 nmol/L with excellent reproducibility. In an APP_NFEV stable SH-SY5Y cellular assay, the EC_{50} for inhibition of EV-Aβ peptide secretion with MRK-3 was 236 nmol/L, consistent with values derived using an EV polyclonal antibody. In an APP_NFEV knock-in mouse model, both Aβ_EV40 and Aβ_EV42 peptides in brain homogenate showed excellent gene dosage dependence. In conclusion, the EV neoepitope specific monoclonal antibody is a novel reagent for BACE1 inhibitor discovery for both *in vitro*, cellular screening assays and *in vivo* biochemical studies. The methods described herein are generally applicable to novel synthetic substrates and enzyme targets to enable robust screening platforms for enzyme inhibitors.

KEYWORDS scFv, antibody, BACE1, amyloid-β precursor protein (APP), immunoassay

INTRODUCTION

Alzheimer's disease (AD) is characterized by the accumulation of toxic amyloid-β (Aβ) peptides in brain extracellular deposits. Amyloid plaques are the primary neuropathological hallmark in AD brains. The Aβ peptide is derived from the sequential cleavage of the amyloid precursor protein (APP) by two aspartyl protease enzymes: β-secretase (BACE) and the γ-secretase complex (Sinha and Lieberburg, 1999; Vassar and Citron 2000). BACE cleavage is thought to be the initial step in the production of Aβ peptides (Cai et al., 2001), since BACE1 knockdown in mice leads to a complete loss of brain Aβ peptide production. BACE knockdown in mice does not affect survival and is associated with minimal adverse effects such as some deficits in myelination during early development (Harrison et al., 2003; Willem et al., 2006). The overall benign phenotype in BACE1 knock out animals has led to the quest for BACE1 inhibitors as a potential therapy for AD.

The endogenous APP site for BACE cleavage (KMDA) shows very low cleavage activity under normal physiological

conditions (Shi et al., 2001; Yang et al., 2003). This observation led to numerous peptide screening efforts to find an optimal cleavage site for BACE inhibitor screening (Tomasselli et al., 2003; Turner et al., 2001). Previously, we reported that an artificial optimized BACE1 cleavage site, where the wild type “KMDA” is mutated to “NFEV,” led to ~100 fold increase in cleavage efficiency by BACE1. The NFEV sequence was also ~10 fold more efficiently cleaved than the “NLDA” sequence derived from familial AD "APP-Swedish" mutation (Shi et al., 2005). Similarly, the replacement of KMDA with SYEV sequence has been shown independently to enhance BACE1 mediated cleavage of substrate (Tomasselli et al., 2003). Therefore, short peptides with the NFEV sequence profoundly increased BACE1 cleavage and enabled the development of robust *in vitro* and cellular screens for identifying BACE1 inhibitors.

In order to screen compound libraries for selective BACE1 inhibitors with the novel NFEV sequence, we have previously described assays to quantify the neoepitope peptides generated following BACE1 cleavage, i.e the NF and the EV products using Western blotting with polyclonal antibodies and mass-spectrometry (MS) (Shi et al., 2005). While, the polyclonal antibody was adequate for the endpoints above, it lacked the sensitivity and specificity required for compound screening efforts. For this purpose, a monoclonal antibody was developed with exquisite specificity and sensitivity to the EV neoepitope.

Here we describe novel monoclonal antibodies specific to the EV neoepitope derived following BACE1 cleavage of either NFEV containing peptides or APP_NFEV mutant protein. The IgG1 antibody was used to develop a sensitive enzyme-linked immunosorbent assay (ELISA) specific for the EV neoepitope derived from both cellular and *in vitro* production of EV peptides. These methods combining identification of enzyme 'super-substrates' and development of companion neoepitope specific antibodies facilitate development of novel enzyme inhibitor screening platforms.

RESULTS

Selection of phage displayed human scFv antibodies specific to EV neoepitope from the CAT libraries

To select EV neoepitope-specific scFv phage, solution based panning was performed using three pooled CAT libraries (BMV, CS and DP47) (Miller et al., 2005; An et al., 2009; Montgomery et al., 2009). The libraries were initially subtracted for phages that bind to the NFEV substrate peptide, followed by panning against EV peptide (EVEFRHDSGYK-biotin) coated magnetic beads (Table 1). Using this strategy, 8 positive phage clones (EV 1–8) were identified in an indirect ELISA that demonstrated binding to the EV neoepitope but not to the intact NFEV peptide (TEEISEVNFEVEFRHDSGYK-biotin) (Fig. 1). Amino acid sequence and complementarity-determining regions (CDRs) of the 8 clones were determined (Fig. 2). All 8 clones had identical amino acid sequence in their heavy chain CDRs. The sequence of light chain CDR regions was identical for clones EV2 and EV6, EV3 and EV7. Due to higher signals with clones EV2 and EV3, these were advanced to additional screening along with EV1, EV4, EV5 and EV8. Thus, a total of 6 distinct phage clones were identified that were specific to the EV neoepitope.

Table 1 Design of biotin labeled peptides

Peptide name	Peptide amino acid sequence
NFEV substrate	SEV**NFEV**EFRHDSGYK-biotin
EV cleavage product	**EV**EFRHDSGYK-biotin
CSP[a] 1 with NF	Biotin-KTEEISEV**NF**
CSP 2 with N	Biotin-KTEEISEV**N**
CSP 3 with FEV	**FEV**EFRHDSGYK-biotin
CSP 4 with NFE	Biotin-KTEEISEV**NFE**
CSP 5 with V	**V**EFRHDSGYK-biotin
CSP 6 with KM	Biotin-KTEEISEV**KM**
CSP 7 with DA	**DA**EFRHDSGYK-biotin
CSP 8 with NL	Biotin-KTEEISEV**NL**

[a] CSP, counter screen peptide.

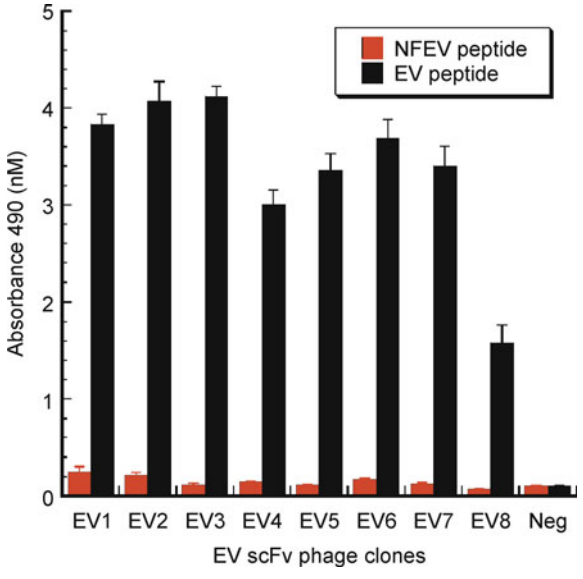

Figure 1. Solution based panning followed by indirect ELISA. Eight scFv clones (EV1–EV8) were identified that bind specifically to the EV peptide but not to the NFEV peptide.

The specificity of the selected phage clones was further confirmed by solid phase indirect ELISA performed using ten different biotin labeled peptides (Table 1) which were synthesized based on the modified amino acid sequence of APP β-secretase cutting site (Fig. 3) — two were the panning


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EV1   AQPAMAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGGSTYYADSVKGRFTISRDNSKN
EV2   AQPAMAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGGSTYYADSVKGRFTISRDNSKN
EV3   AQPAMAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGGSTYYADSVKGRFTISRDNSKN
EV4   AQPAMAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGGSTYYADSVKGRFTISRDNSKN
EV5   AQPAMAEVQLLESGGGLVQPGGSLRLSCAASEFTFSSYAMSWVRQAPGKGLEWVSAISGSGGGSTYYADSVKGRFTISRDNSKN
EV6   AQPAMAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGGSTYYADSVKGRFTISRDNSKN
EV7   AQPAMAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGGSTYYADSVKGRFTISRDNSKN
EV8   AQPAMAEVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGGSTYYADSVKGRFTISRDNSKN
                          VH-CDR1                    VH-CDR2
EV1   TLYLQMNSLRAEDTAVYYCARYYCPNGVCNSFDYWGRGTMVTVSSGGGGSGGGGSGGGGSAQS-VLTQPPSASGTPGQRVTIS
EV2   TLYLQMNSLRAEDTAVYYCARYYCPNGVCNSFDYWGRGTMVTVSSGGGGSGGGGSGGGGSALSYVLTQPPSASGTPGQRVTIS
EV3   TLYLQMNSLRAEDTAVYYCARYYCPNGVCNSFDYWGRGTMVTVSSGGGGSGGGGSGGGGSAQS-VLTQPPSASGAPGQRVTIS
EV4   TLYLQMNSLRAEDTAVYYCARYYCPNGVCNSFDYWGRGTMVTVSSGGGGSGGGGSGGGGSAQS-VLTQPPSASGTPGQRVTIS
EV5   TLYLQMNSLRAEDTAVYYCARYYCPNGVCNSFDYWGRGTLVTVSSGGGGSGGGGSGGGGSAQS-VLTQPPSASGTPGQRVTMS
EV6   TLYLQMNSLRAEDTAVYYCARYYCPNGVCNSFDYWGRGTMVTVSSGGGGSGGGGSGGGGSALSYVLTQPPSASGTPGQRVTIS
EV7   TLYLQMNSLRAEDTAVYYCARYYCPNGVCNSFDYWGRGTMVTVSSGGGGSGGGGSGGGGSAQS-VLTQPPSASGAPGQRVTIS
EV8   TLYLQINSLRAEDTAVYYCARYYCPNGVCNSFDYWGKGTMVTVSSGGGGSGGGGSGGGGSALSYVLTQPPSASGTPGQRVTIS
                          VH-CDR3                    Linker

EV1   CSGSSSNIGSNTVNWYQQLPGTAPKLLIYSNNQRPSGVPDRFSGSKSGTSASLAISGLQSEDEADYYCAAWDDSLNGGVFGGGTK
EV2   CSGSSSNIGSNTVNWYQQLPGTAPKLLIYTNDQRPSGVPDRFSGSKSGTSASLAISGLRSEDEAHYYCAAWDDSLSALVFGEGTK
EV3   CSGSSSNIGSNTVNWYQQIPGRAPKLLIYSDNERPSGVPDRFSGSKSGTSASLAISGLQSEDEADYYCAAWDDSLKRGLFGGGTK
EV4   CSGSSSNIGSNTVNWYQQLPGTAPRLLIYSNNQRPSGVPDRFSGSKSGTSASLAISGLQSEDEADYYCAAWDDSLNGYVFGTGTK
EV5   CSGSSSNIGSNTVNWYQQLPGTAPKLLIYSNSQRPSGVPDRFSASKSGTSASLAISGLQSEDEADYYCAVWDDSLNGLIFGGGTK
EV6   CSGSSSNIGSNTVNWYQQLPGTAPKLLIYTNDQRPSGVPDRFSGSKSGTSASLAISGLRSEDEAHYYCAAWDDSLSALVFGEGTK
EV7   CSGSSSNIGSNTVNWYQQIPGRAPKLLIYSDNERPSGVPDRFSGSKSGTSASLAISGLQSEDEADYYCAAWDDSLKRGLFGGGTK
EV8   CSGSSSNIGSNTVNWYQQIPGTAPKLLIYSNNQRPSGVPDRFSGSKSGTSASLAISGLQSDDEATYYCAAWDDSLTGVVFGGGTK
        VL-CDR1                    VL-CDR2                              VL-CDR3
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Figure 2. Comparison of amino acid sequences of the 8 identified EV-specific scFv clones. Amino acid differences in complementary determining regions (CDR) 1, 2 and 3 of both heavy and light chains are bold and underlined. CDR portions in both heavy and light chains among the 8 clones are aligned and underlined. The amino acid sequence in CDR region shows that clone EV2 is the same as EV6 and EV3 is identical to EV7.

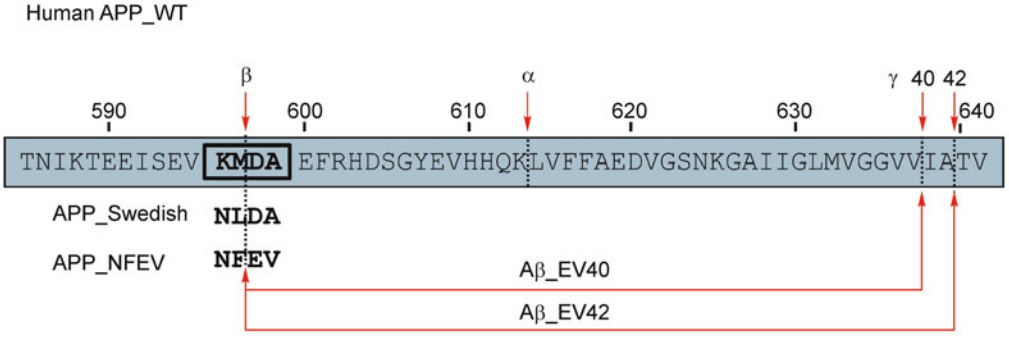

Figure 3. Schematic diagram of modified partial human APP amino acid sequence and α- ,β-, and γ-secretase cutting sites. The human APP_WT BACE cleavage site is indicated by KMDA, while the Swedish mutation is NLDA and the artificial BACE substrate is the NFEV sequence. The secretase cleavage sites are indicated by arrows. α, alpha-secretase; β, beta-secretase; γ, gamma-secretase. Cleavage of the APP_NFEV sequence by β-secretase followed by γ-secretase leads to the production of Aβ_EV40 and Aβ_EV42 peptides.

peptides (NFEV substrate and EV cleavage product) and eight were counter-screening peptides (CSP) with either the C-terminal amino acids of N, NF, NFE, KM, NL or the N-terminal amino acids of FEV, V and DA, respectively. All the selected clones were specific to the N-terminal amino acid E (Fig. 4). These data confirmed the binding selectivity and specificity of the EV scFv phage-displayed antibodies. Based on the degree of binding to the EV peptides and signal to noise ratio of these clones (S/N ratios ~32, 35, 48, 40 and 23 for clone EV1, EV2, EV3, EV5 and EV8, respectively), phage scFv clone EV3 was selected for further characterization.

Expression and characterization of full-length human IgG1 antibody

The EV3 scFv was converted to an IgG1 antibody to improve

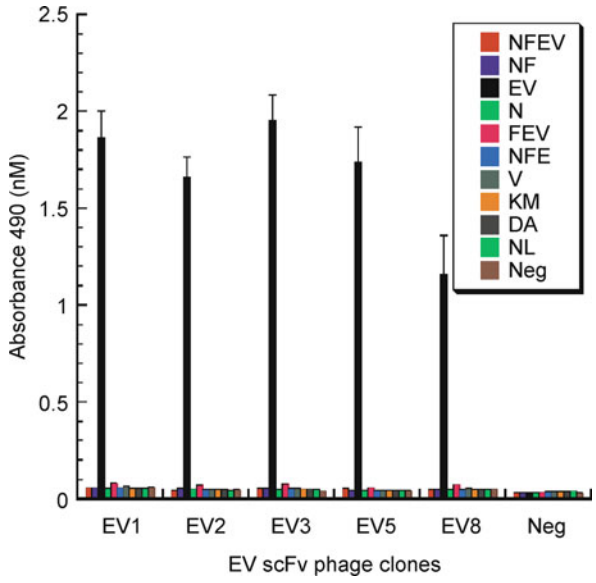

Figure 4. Specificity of the phage scFv clones to the EV neoepitope. The identified EV-specific phage clones were counter-screened using an array of peptides (Table 1) to assess their specificity for binding to the N-terminal EV peptide. All 5 identified EV reactive clones (EV1, EV2, EV3, EV5 and EV8) were highly specific to the N-terminal EV peptide.

its stability and affinity for the EV neoepitope peptide (Miller et al., 2005; An et al., 2009; Montgomery et al., 2009; Palys et al., 2006). scFv heavy (H) and light (L) chain cDNA was cloned into human IgG1 expression vectors PEU1.2 and PEU4.2, respectively (Miller et al., 2005; Montgomery et al., 2009). The H and L chain DNA plasmids were co-transfected transiently into the HEK293-EBNA cell line and the purified EV3 IgG antibody was characterized for purity and integrity. A single band at 150 kDa is detected under non-denaturing condition and two bands at ~50 kDa and ~22 kDa are detected under β-ME denaturing conditions, indicating heavy and light chains respectively (Data not shown). These data indicate the successful cloning, expression and purification of the EV3 IgG1 molecule.

The EV3 antibody (EV3-IgG1) was conjugated to alkaline phosphatase (AP) to further characterize its properties and to develop assays to detect the EV-neoepitope in BACE1 *in vitro* enzymatic assays. EV3-IgG1 maintains its specificity for the EV epitope compared to other counter-screening peptides following conversion from scFv displayed on phage to IgG1. EV3-IgG1-AP also shows excellent sensitivity in binding the N-terminal EV-neoepitope with a signal to noise ratio of ~6000 (Fig. 5A). In contrast, the purified rabbit anti-EV polyclonal antibody had poorer selectivity for binding to the different screening peptides (Fig. 5B). EV3-IgG1-AP was then tested against a range of concentrations of EV biotinylated peptide. EV3-IgG1-AP shows a dose dependent decline in signal with a lower limit of reliable quantitation (LLRQ) of 0.31 nmol/L for the peptide (Fig. 5C).

In vitro BACE1 enzymatic assay with NFEV peptide substrate and detection using EV3-AP antibody

The BACE1 enzymatic reaction consists of BACE1-dependent cleavage of an "NFEV" peptide substrate with C-terminal biotin, leading to the generation of the EV-product (Shi et al., 2005). The EV-product is captured onto a streptavidin-coated plate and detected directly using the EV3-IgG1-AP. The EV-product is detected in reactions with BACE1 concentrations as low as 40 pmol/L (Fig. 6A). A linear decline in EV-product signal was observed as a function of decline in BACE1 concentration ($r = 0.99$, $p < 0.001$). In addition, we observed a time-dependent linear increase in EV-product over a 3 h time period at a fixed BACE1 reaction concentration of 0.3 nmol/L (Fig. 6B). These data indicate that the AP-conjugated EV3-IgG1 can be used to reliably quantify BACE1 cleavage products and thus enable evaluation of BACE1 enzyme structure and activity relationships for small molecule drug screening.

Assessment of BACE1 inhibitor potency using *in vitro* enzyme and cell culture systems

We tested the ability of a well characterized BACE1 inhibitor MRK-3 (Stachel et al., 2004; Wu et al., 2008) to inhibit BACE1 activity in an *in vitro* BACE1 enzymatic assay using the NFEV-biotinylated substrate. The BACE1 enzymatic assay consists of a reaction using 0.3 nmol/L of BACE1 enzyme along with 300 nmol/L NFEV substrate for 2 h at 37ºC. Typically, ~3% of the substrate is cleaved producing approximately 10 nmol/L of the EV-product, determined using titration with a synthetic biotin-labeled EV peptide standard curve. A range of concentrations of MRK-3 were tested and the EV-product was detected using EV3-IgG1-AP. MRK-3 showed a dose-dependent inhibition of EV-production in this assay, with an IC_{50} of 2.4 nmol/L (Fig. 6C). These data are consistent with results using the EV rabbit polyclonal antibody (data not shown) and with results previously reported for MRK-3 (Stachel et al., 2004; Pietrak et al., 2005). This assay also showed excellent reproducibility and stability with intra-day and inter-day variation of 12% and 11%, respectively (Table 2).

We next evaluated this assay to test the impact of the BACE1 inhibitor MRK-3 in cells. We have previously reported that SH-SY5Y cells stably expressing APP_NFEV show robust BACE1 cleavage and secretion of EV_Aβ peptides (Shi et al., 2005). APP_NFEV expressing SH-SY5Y cells were grown in a 96 well dish overnight. The culture medium was then replaced with a range of concentrations of MRK-3 and conditioned medium was collected 24 h after treatment. A dose-dependent reduction in EV_Aβ was observed with an

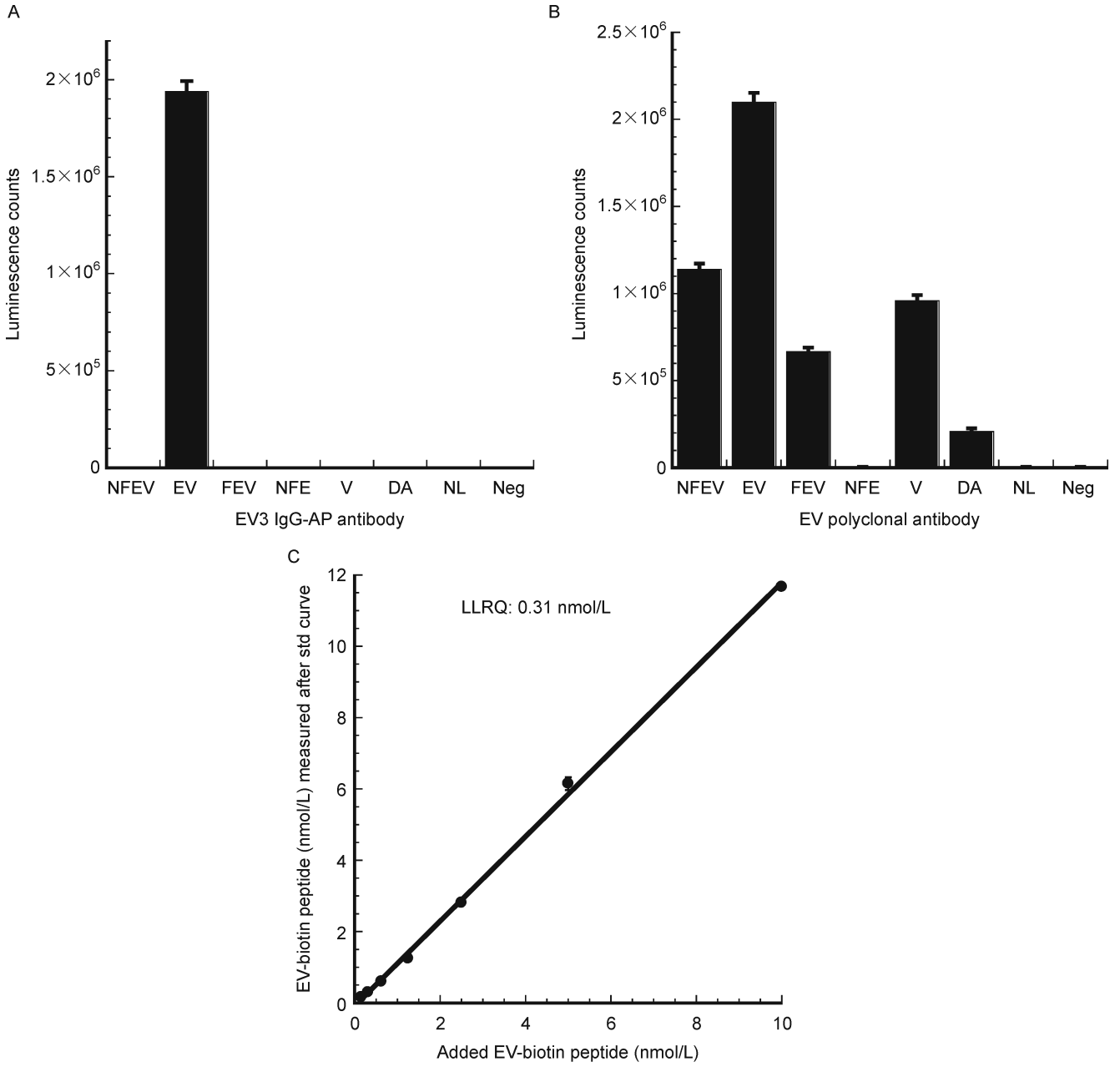

Figure 5. EV3-IgG1 binding specificity and sensitivity. (A) EV3-IgG1 shows robust signal with the EV N-terminal peptide and greater than 6000-fold signal to noise ratio without detectable cross reactivity to other N or C-terminal modified peptides. (B) The EV rabbit polyclonal antibody showed significant reactivity with other counter-screening peptides in addition to the EV N-terminal peptide. (C) Sensitivity for the detection of synthetic EV N-terminal peptide by EV3-IgG-AP. The lower limit of reliable quantitation of the EV peptide is ~0.3 nmol/L.

EC_{50} of 236 nmol/L for MRK-3 (Fig. 7A), using Aβ capture with the mid-domain antibody 4G8, and detection with EV3-IgG1-AP. This data is consistent with the EV_Aβ EC_{50} obtained in a sandwich ELISA using the EV polyclonal antibody as a detecting antibody (EC_{50} = 254 nmol/L) (Fig. 7B). BACE1 inhibition is associated with an increase in α-secretase processing of APP and consequent generation of sAPPα. In the same experiment as above, a dose-dependent increase in sAPPα was observed, similar to that previously reported in mouse brain samples (Sankaranarayanan et al., 2008) and in primary neuron culture (Wu et al., 2011). This dose-dependent increase in sAPPα suggests that the decline in EV_Aβ following BACE1 inhibition is not due to cellular toxicity. The EC_{50} for sAPPα elevation was 332 nmol/L

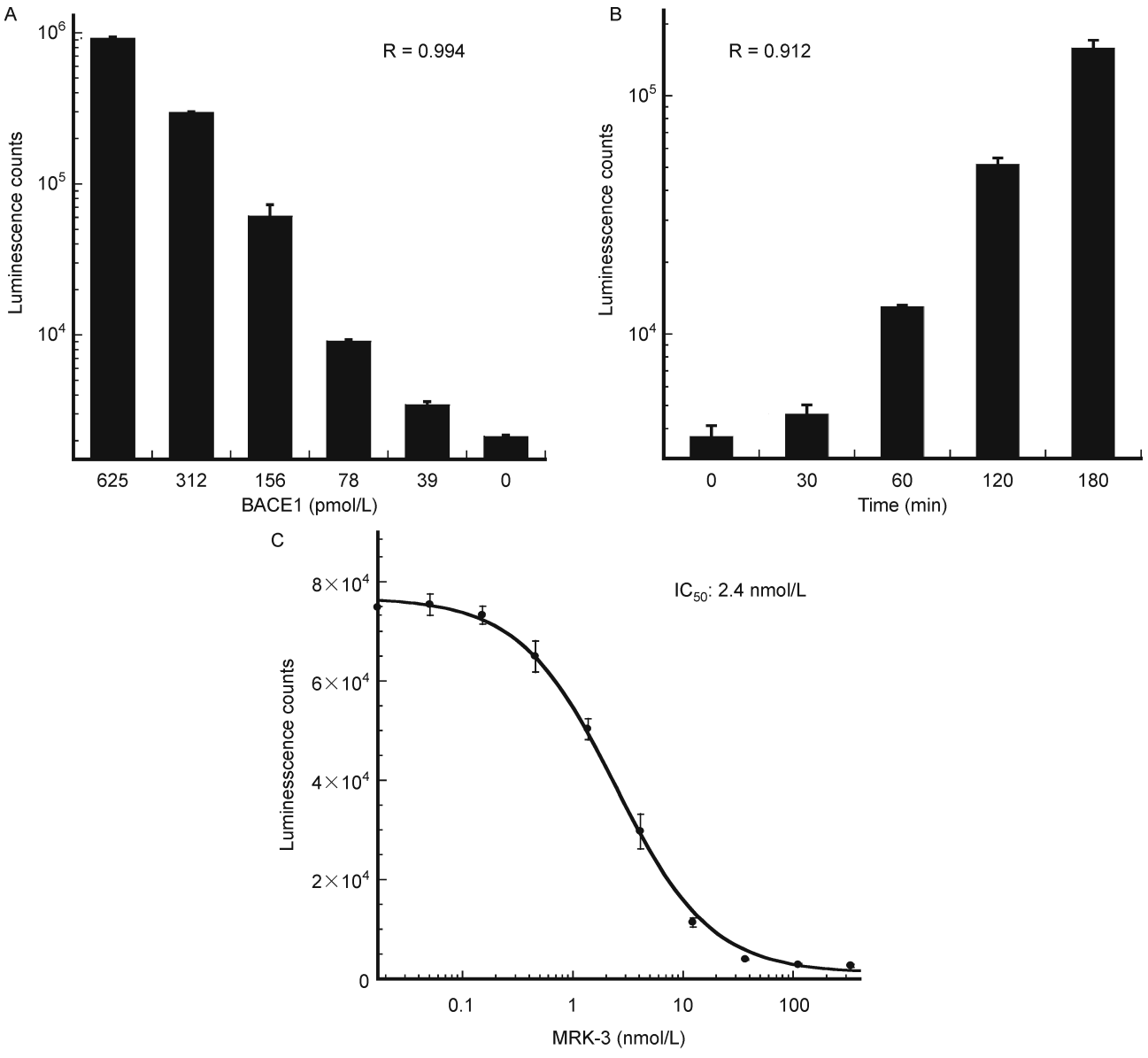

Figure 6. ***In vitro*** **characterization of EV3-IgG1 antibody in a BACE1 enzymatic assay.** (A) BACE1 concentration response in an *in vitro* BACE1 enzymatic reaction using NFEV substrate, followed by detection of the EV N-terminal peptide with EV3-IgG1-AP. A linear dose-dependent increase in EV peptide signal is observed as a function of BACE1 concentration ($r = 0.99$, $p < 0.001$, $n = 3$). (B) Time course of production of EV N-terminal peptides following cleavage of NFEV substrate by 0.3 nmol/L BACE1 in an *in vitro* enzymatic reaction. A linear increase in EV N-terminal peptide signal was observed over time ($r = 0.912$, $p < 0.001$, $n = 3$). (C) A specific BACE1 inhibitor MRK-3 was tested at a range of concentrations in the *in vitro* BACE1 enzymatic reaction with NFEV peptide substrate. A dose-dependent inhibition of EV N-terminal peptide production was observed with MRK-3 with an IC_{50} of 2.4 nmol/L.

Table 2 IC_{50} for BACE1 inhibitor MRK-3 using NFEV substrate ($n = 5$)

	Intra-day	Inter-day
Mean (nmol/L)	2.11	2.14
SD (nmol/L)	0.26	0.24
Min	1.80	1.81
Max	2.29	2.40
% CV	12.39	11.17

IC_{50}, half maximal inhibitory concentration; CV, coefficient of variation.

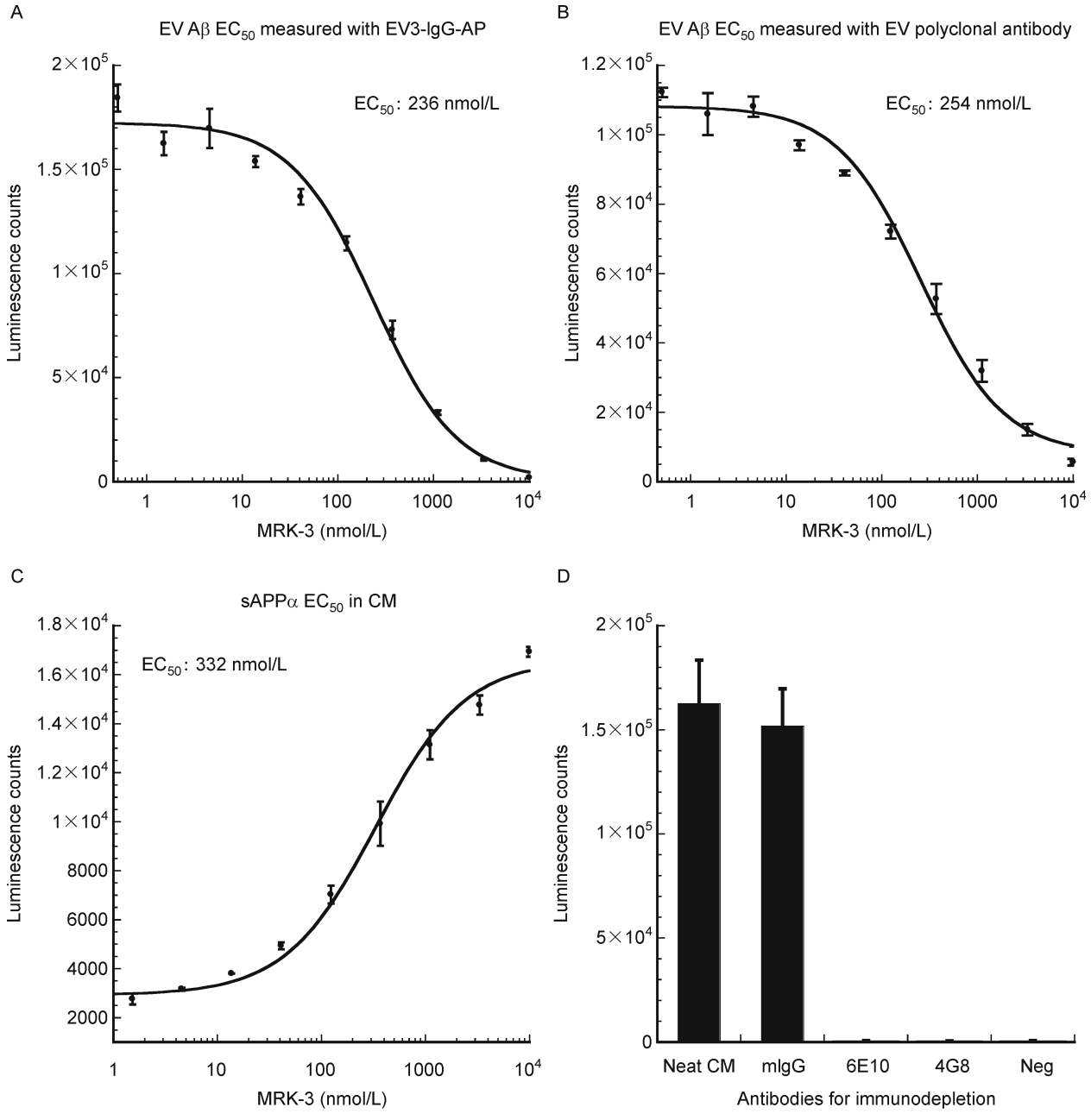

Figure 7. Characterization of the EV3-IgG1 antibody in a cellular assay for assessing BACE1 inhibitors. (A) Production of EV_Aβ peptides from SH-SY5Y cells stably expressing APP_NFEV was tested at a range of concentrations of MRK-3. EV_Aβ peptides were measured using the detecting antibody EV3-IgG1-AP in a sandwich ELISA following capture with 4G8. A dose-dependent inhibition of EV-Aβ production was observed with an EC_{50} of ~236 nmol/L. (B) Measurement of EV_Aβ peptides using an EV rabbit polyclonal detecting antibody yielded comparable EC_{50} of ~254 nmol/L in the same experiment as in (A). (C) MRK-3 treatment led to a dose-dependent increase in sAPPα (EC_{50} ~332 nmol/L) from the same experiment as in (A), indicating no cellular toxicity. (D) Assay specificity in EV_Aβ detection with EV3-IgG1-AP antibody. Immunodepletion of Aβ peptides with Aβ-selective antibodies 6E10 or 4G8, but not mouse IgG control antibody led to a complete loss of signal in the EV3-IgG1-AP detection system.

(Fig. 7C). Specificity for detection of EV_Aβ was assessed by immunodepletion with either 6E10 or 4G8 (anti-Aβ antibodies) or with control mouse IgG coated beads following overnight incubation. We observed no detectable level of EV_Aβ in conditioned medium following 6E10- or 4G8-immunodepletion, and no significant decline in signal with control mouse IgG (Fig. 7D). These data taken together indicate that EV3-IgG1 is specific for the EV-neoepitope

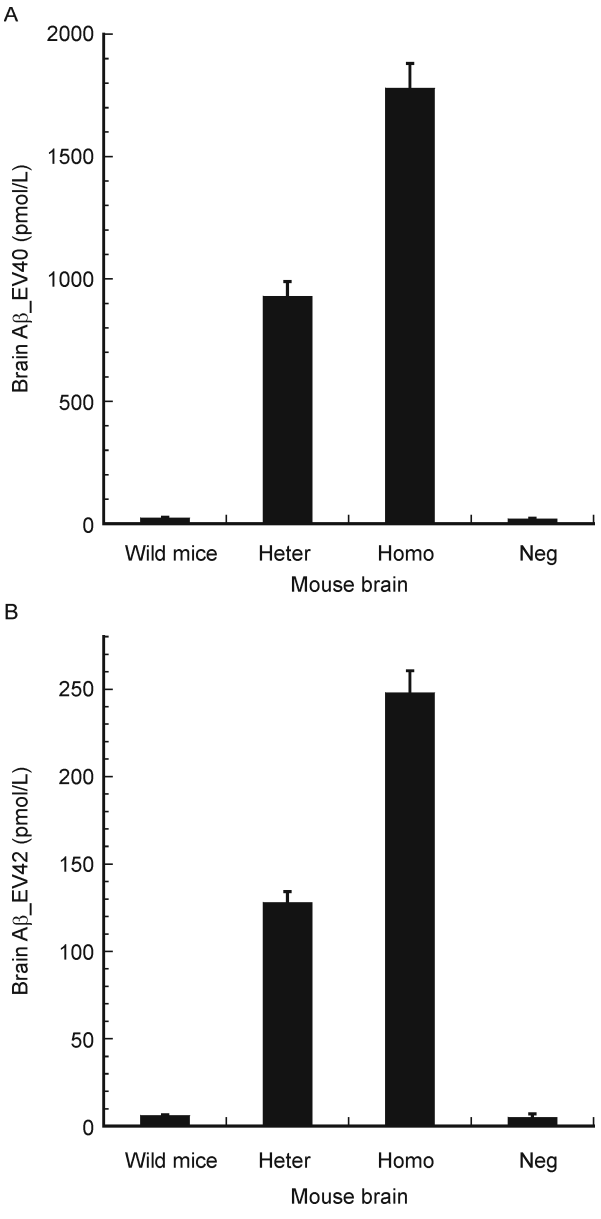

Figure 8. Brain Aβ_EV40 and Aβ_EV42 in APP_NFEV mice at 6 months. Brain Aβ gene-dosage dependence, with homozygote (Homo) displaying 2 fold higher levels than heterozygote (Heter) litter mates. Wild type animals show no detectable signal. (A) Aβ_EV40 and (B) Aβ_EV42 levels. (n = 3, Mean ± SEM).

generated following BACE1 cleavage of the APP_ NFEV substrate and can be effectively used in cellular assays for screening compounds.

Aβ_EV40 and Aβ_EV42 in APP_NFEV mice brain

The APP_NFEV knock-in transgenic mice have a targeted replacement of endogenous "KMDA" BACE1 cleavage site with the "NFEV" sequence to enhance BACE1 mediated cleavage of APP, along with humanizing mutations at positions 5, 10 and 13 of mouse Aβ sequence (Simon et al., 2004; McCampbell et al., 2011). Brain Aβ_EV40 and Aβ_EV42 levels were measured in APP_NFEV heterozygous and homozygous mice by ELISA assay similar to that described previously (Sankaranarayanan et al., 2008), but in this case, EV3-IgG1 was used as a capture antibody, followed by detection with the AP-conjugated Aβ40 and 42-specific antibodies. Brain Aβ_EV40 levels were 930 pmol/L and 1780 pmol/L in heterozygous and homozygous 6 month old mice, respectively (Fig. 8A). In contrast, brain Aβ_EV42 levels were 128 pmol/L and 248 pmol/L in heterozygous and homozygous mice, respectively (Fig. 8B). Wild type littermates show no signal since they express the DA_Aβ peptides that are not detected by the EV-specific antibodies. The ratios of Aβ_EV42 to Aβ_EV40 were 13.8% and 13.9% for heterozygous and homozygous mice, respectively. These results indicate gene-dosage dependence on the levels of Aβ measured in the APP_ NFEV mice. In conclusion, the EV specific monoclonal antibody would enable robust measurement of APP metabolites in the APP_NFEV mice to characterize their pathophysiology and also serve as a useful pharmacodynamic endpoint for evaluating potential Aβ lowering agents *in vivo*.

DISCUSSION

Here, an optimized ELISA assay for screening a compound library to generate inhibitors of the BACE enzyme is reported. Previously, a preferred BACE substrate was identified (Shi et al., 2005), and using the amino acid sequences of the BACE neoepitope as bait, a scFv phage-displayed antibody, specific to the N-terminal EV neoepitope cleavage product was identified. The EV-specific scFv was identified from the CAT libraries and converted to an IgG1 monoclonal antibody. The selected phage antibody clone EV3 binds specifically to the peptide neoepitope, and shows no cross reactivity with the uncleaved NFEV substrate, the natural KMDA or Swedish mutation NLDA substrate sequence, their respective cleavage products, or those containing single N-terminal amino acid modifications. The EV3 IgG1 antibody has greatly improved selectivity over the previously characterized rabbit EV neoepitope polyclonal antibody. Its binding sensitivity (0.3 nmol/L) and its selectivity (> 6000-fold) make it an excellent reagent for development of BACE1 inhibitor assays.

The EV3 IgG1 antibody enabled a robust, sensitive ELISA assay to detect BACE1 mediated APP_NFEV cleavage products in both *in vitro* and cellular assays. Previous *in vitro* BACE1 screening assays have used either fluorescence resonance energy transfer (FRET) readouts, where inhibition of substrate cleavage leads to a loss of FRET transfer and a decline in signal, or direct detection of the cleavage products by high-performance liquid chromatography (HPLC) or MS

(Shi et al., 2005). The limits of FRET-based assay include non-selectivity and false-positive signals from intrinsic compound fluorescence, while the HPLC and MS methods had low throughput. BACE1-mediated NFEV cleavage products were also evaluated by Western blotting and MS in APP_NFEV over-expressing cells (Shi et al., 2005), but these semi-quantitative methods lack throughput for effective screening (Tomasselli et al., 2003). In addition, the high concentrations of BACE enzyme required to robustly detect cleavage products in some of the aforementioned assay formats preclude their use for identification of sub-nmol/L potency inhibitors (Copeland, 2005). In contrast, the greater affinity of EV3-IgG1 for the EV-neoepitope enabled detection of BACE1 cleavage products at concentrations of BACE1 less than 50 pmol/L. The EV3-IgG1 based assay also demonstrated excellent reproducibility (% CV of < 12% in both intra- and inter-day assessments). Because EV3-IgG1 is a recombinant human antibody, it can be produced in a virtually unlimited supply with minimal lot to lot variation over time.

In cell-based assays and human cerebral spinal fluid, MS has revealed many N-terminal truncated forms of Aβ species (Rüfenacht et al., 2005). Thus, use of an N-terminal specific detection antibody is critical to reliably measure a specific BACE1 cleavage product. Assays employing EV3-IgG1 and a BACE1 inhibitor (MRK-3) generated IC_{50} and EC_{50} values comparable to previous reports (Stachel et al., 2004; Pietrak et al., 2005), where a rabbit polyclonal EV antibody was used. In addition we show that the EC_{50} values for EV-Aβ lowering correlate with sAPPα elevations following BACE1 inhibition in cell based assays. The EV3 antibody also enabled effective measurement of EV-Aβ species from brain extracts from the APP_NFEV mice. These methods would enable further characterization of the mouse model and pharmacodynamic assays for Aβ lowering agents.

In conclusion, we selected and characterized EV3, a phage clone specific to the EV neoepitope following BACE1 cleavage of an artificial NFEV peptide substrate. The EV3-IgG1 antibody retains this specificity and enabled a robust immunoassay for detecting N-terminally cleaved EV-Aβ peptides. This antibody facilitates a robust screening platform for novel BACE1 inhibitors including *in vitro*, cell-based and *in vivo* studies.

MATERIALS AND METHODS

Peptides

Table 1 shows the 10 custom designed biotin labeled peptides used for panning, screening and counter screening assays (American Peptide Company, Sunnyvale, CA). These included related peptides grouped (1) the novel BACE "NFEV" substrate sequence, BACE1 cleavage products with C-terminal "NF" and N-terminal "EV" peptides, single amino acid changes at the end of the peptide (NFE, V, N, FEV), (2) BACE1 wild-type substrate KMDA cleavage products with C-terminal KM and N-terminal DA, and (3) the Swedish mutation C-terminal NL peptide as shown in Fig. 3. The NFEV and EV peptides used for solution-based panning were bound to streptavidin coated Dynabeads M-280 (Invitrogen, Cat#112-05D). The beads were incubated with peptides at 100 nmol/L of final concentration for 1 h at room temperature and washed according to manufacturer's instructions.

Selection of anti-EV neoepitope specific scFv phage antibody

Three phage displayed scFv libraries (BMV, CS and DP-47) were obtained from Cambridge Antibody Technologies (CAT, Cambridge, United Kingdom) (Miller et al., 2005; An et al., 2009; Montgomery et al., 2009). The pooled libraries were panned in a 3-step procedure based on methods described (Vaughan et al., 1996). First, the libraries were deselected against spanning NFEV peptide-bead complexes for 3 h at 4°C and the unbound supernatant was collected. The deselected phages were then panned against neoepitope EV peptide-bead complexes for 3 h at 4°C. The beads were washed three times with 50 mmol/L, pH 7.4 phosphate buffer with 0.2% Tween 20 (PBST) and the bound phages were eluted with 0.2 mL of 0.1 mol/L Triethylamine (pH 11) buffer and neutralized with 0.1 mL of 1 mol/L Tris buffer at pH 7.5. The eluted phages were transformed into *E. coli* strain TG1, plated on agar and incubated at 37°C overnight. The colonies were collected and a second round of panning against EV peptide-bead complexes was performed as above. At the end of this round of panning, phage-infected TG1 cells were serially diluted and plated for single colony identification on tryptone-yeast extract (TY)-agar containing 100 μg/mL ampicillin and kanamycin. Twenty individual clones from each plate were randomly picked and grown at 37°C overnight in "Terrific broth" (TB) containing 100 μg/mL kanamycin and rescued with M13K07 helper phage. The amplified phage culture supernatant from each clone was collected and tested by indirect ELISA.

Phage ELISA screens

Streptavidin-coated 96 well plates (Thermo Scientific, Cat#15503) were incubated with 100 μL of 50 nmol/L biotin labeled peptide (either NFEV or EV) at room temperature for 1 h and washed three times with PBST. Culture supernatant containing the cloned phage was diluted 100 fold in 3% BSA/PBS, added to the plates and incubated at room temperature (RT) for 1 h. The plates were washed three times with PBST and reacted with anti-M13 phage antibody conjugated with horseradish peroxidase (HRP) at 1:5000 dilution in 3% BSA/PBS with 0.05% Tween 20 for 1 h at RT. Following three washes with PBST, HRP substrate 3,3',5,5'-tetramethylbenzdine (TMB, Thermo Scientific, Cat#34021) was added for 20 min and the color reaction was stopped with 50 μL of 2 mol/L H_2SO4. Absorbance at 490 nM was measured with a SpectraMax plate reader (Molecular devices). Clones that bound to EV but not to the intact substrate peptide NFEV were selected for further characterization.

The EV specific clones were counter-screened with the eight additional biotin labeled peptides (Table 1) for specificity using the same procedure as above. scFv phage clones specific to peptide with N-terminal amino acid E were selected for DNA extraction and sequencing of both the scFv frame region (FR) and CDRs using the sequencing oligonucleotide primers from CAT as previously

described (Miller et al., 2005; An et al., 2009; Montgomery et al., 2009).

Conversion of scFv phage into full-length human IgG1 anti-EV antibody

ScFv clone EV3 was selected for conversion into an IgG1 molecule. The VH and VL domains of EV3 scFv were cloned by PCR using primers based on the sequence located at each end of linkers for both heavy and light chains. The primers for the VH chain contained restriction sites of *Bss*HII at the 5' end (AF14, CTCTCCA-CAGGCGCGCACTCC**GAGGTGCAGCAGCTGTTGGAG**) and *Bsm*B1 at the 3' end (RH85, TGGAGTACC**TGAGGAGACGGTGAC-CATTGTCCC**). The primers for the VL chain contained restriction sites of *Apa*LI at the 5' end (AF31, CTCTCCACAGGCGTGCACTCC-**CAGTCTGTGCTGACTCAGCC**) and *Pac*I at the 3' end (AF26, CTATTCCTTAATTAAGTTAGATCTATTCTGACTCACC**TAG-GACGGTGACCTTGGTCCCTCC**). PCR products for the VH chain were digested with *BSS*HII and *Bsm*B1 at 50°C for 90 min and those for VL chain were digested with *Apa*LI and *Pac*I at 37°C for 90 min, respectively. The enzyme digested products were gel-purified and ligated with the expression vectors PEU1.2 for VH and PEU4.2 for VL lambda (CAT) containing a human gamma 1 heavy or light chain expression cassette, respectively, NEO (G418R) selectable marker for mammalian cells, and ampicillin resistant marker for *E. coli* cells. Both plasmid DNAs were purified using Qiagen Midi-purification kit and sequenced with universal vector sequencing primers as previously described (Miller et al., 2005; An et al., 2009; Montgomery et al., 2009). The inserts for heavy and light chains were also confirmed by digestion with restriction enzymes. For the expression of soluble antibodies in mammalian cells, both VH and VL plasmid DNAs were transiently co-transfected into human HEK293-EBNA cell lines with FuGene 6 (Roche Applied Science, Cat# 05061377001) as described previously (An et al., 2009; Montgomery et al., 2009). Transiently transfected cells were maintained in HEK293 serum-free medium for 10 d. During this time, the medium was changed twice and culture supernatants were collected and filtered with 0.22 μm filter. The IgG1 antibody was purified with Protein A beads, eluted with pH 4.5, 0.1 mol/L sodium acetate buffer and neutralized with 10% volume of 1 mol/L Tris buffer at pH 7.4. The purified antibody was dialyzed against PBS and stored frozen at −80ºC until use.

Characterization and alkaline phosphatase conjugation of EV-specific IgG1 antibody

Purified EV3-IgG was resolved on a 10% SDS-PAGE gel with or without 5% β- mercaptoethanol (β-ME) in sample buffer and stained with Coomassie blue to determine molecular weight under denaturing and non-denaturing conditions. To further characterize EV3-IgG1, it was conjugated directly with alkaline phosphatase (AP) (KPL, Gaithersburg, MD, Cat # 85-00-02) according to the manufacturer's instruction, and stored at −20°C with an equal volume of 100% glycerol.

AP conjugated EV3-IgG1 was tested for binding specificity, sensitivity against synthetic peptides and EV peptides derived from *in vitro* BACE1 enzymatic reaction with a spanning NFEV peptide. The binding specificity was compared with a purified EV polyclonal antibody which was generated in rabbits immunized with KLH linked at N-terminal five amino acids of EV Aβ1-40 (EVEFRC-KLH) peptide. Fifty nmol/L of biotin-labeled peptides were captured on a streptavidin coated plate, washed and blocked with 3% BSA/PBS, 100 μL of 0.1 μg/mL AP conjugated EV3 antibody was added and incubated at RT for 1 h. The plate was then washed with PBST five times and developed with AP substrate (Applied Biosystem, Cat# T-2214) (Wu et al., 2008). Luminescence was counted in Envision 2104 multi-label reader (PerkinElmer, CA). For comparison, an indirect ELISA was applied for EV polyclonal antibody in which 1:3000 diluted rabbit polyclonal antibody at a final concentration of 0.1 μg/mL was added to the plate and followed by detection with a goat anti-rabbit IgG-AP (Wu et al., 2008).

Binding sensitivity of EV3 scFv and EV3-IgG was performed against EV-biotin peptide. A range of concentrations (10–0.08 nmol/L for EV3-IgG1-AP and 126–4 nmol/L for scFv displayed on phage) of biotinylated EV peptide was captured on streptavidin plate. After washing and blocking, 100 μL of phage supernatant at 1:500 dilution was added overnight at 4ºC. Plates were then washed three times with PBST and secondary anti-M13-HRP antibody was added at 1:5000 in 3% BSA/PBS with 0.05% Tween 20 for 1 h and developed as above. To test the EV3-IgG antibody, 50 μL of EV3-AP in 3% BSA/PBS with 0.05% Tween 20 was added at 0.1 μg/mL onto peptide bound plate, incubated overnight, washed and developed as above. EV3 antibody sensitivity was quantified using the lowest limit of reliable quantitation (LLRQ), estimated as the lowest peptide concentration with % CV of less than 20%.

To characterize EV3 binding to EV peptides generated from an *in vitro* BACE1 enzymatic reaction, a 15-mer NFEV spanning peptide containing a C-terminal biotin (SEVNFEVEFRHDSGYK-biotin) was reacted with BACE1 enzyme *in vitro*. BACE1 dose-response properties were defined using the NFEV peptide at 300 nmol/L incubated with different concentrations of BACE1 enzyme for 2 h at 37ºC. Time course studies were performed using the NFEV-peptide incubated with 0.3 nmol/L BACE1 enzyme for different times ranging from 0–3 h. The BACE1 cleavage product (EVEFRHDSGYK-biotin) was quantified using EV3-IgG1-AP as described above.

Validation of the *in vitro* BACE1 inhibitor assay

The BACE1 enzymatic activity assay was modified from methods previously described (Shi et al., 2005; Pietrak et al., 2005), while the cleavage products were quantified as described herein. Briefly, baculovirus expressed and purified truncated BACE1 (aa 1–460) (Shi et al., 2001) at 0.3 nmol/L was incubated at a range of concentrations with a previously characterized BACE1 inhibitor MRK-3 (Stachel et al., 2004; Sankaranarayanan et al., 2008; Wu et al., 2011) for 30 min at RT. After this preincubation, the BACE1 substrate (15 amino acid peptide with C-terminal biotin) was added at a final concentration of 300 nmol/L to the reaction buffer (50 mmol/L NaOAc, 0.01% BSA, 15 mmol/L EDTA, 0.2% CHAPS, 1 mmol/L Deferoxamine Mesylate and 10 μmol/L pepstatin A at pH 4.5) and incubated for 2 h at 37°C in a shaker at 40 rpm (Wu et al., 2008). The reaction was quenched using 50 μL of 1 mol/L Tris buffer (pH 8.0). The BACE1 cleavage product was quantitated with EV3-AP antibody as described above. For comparison, cleavage products were also quantified using the EV polyclonal antibody. The half maximal inhibitory concentration (IC_{50}) was calculated using non-linear curve fitting. The intra-day and inter-day coefficient of variation (% CV) was then determined for the EV3 antibody signal.

Cell culture analysis of APP_NFEV processing products

A human APP construct containing the NFEV mutation at the BACE1 cleavage site (APP_NFEV) was transfected into SH-SY5Y cell line and cultured using conditions similar to that previously described (Shi et al., 2005). Cells were incubated with BACE1 inhibitor MRK-3 at a range of concentrations overnight in conditioned medium (CM). A modified EV_Aβ assay was used to characterize the cleavage products in which 50 µL of diluted CM in 3% BSA/PBS following MRK-3 treatment was mixed with 50 µL of 3 µg/mL biotin-labeled 4G8 antibody, which recognizes Aβ amino acid 17 to 24 (Covance, Cat# 9240-10) in 3% BSA/PBS. Following this step, 50 µL of 1 µg/mL EV3-IgG1-AP (in 3% BSA/PBS with 0.3% tween 20) was added and incubated at RT for 3 h. This mixture was then transferred onto a streptavidin-coated 96 well plate for 2 h. The plate was washed with PBST five times and then developed using AP substrate. EV_Aβ product in CM was also detected with the EV polyclonal antibody by adding 2 µg/mL of the EV polyclonal antibody instead of EV3-IgG1-AP and detected with a goat anti-rabbit IgG-AP (Bio-Rad, Cat#170-6518) at 1:10,000 dilution in 0.1% tween-casein blocking buffer (Thermo Scientific, Cat# 37528) for 1 h at RT. The plate was washed and developed as above. The half maximal effective concentration (EC_{50}) for inhibition of EV_Aβ peptide secretion was calculated using a non-linear curve fitting in Kaleidagraph. To confirm cell viability and efficacy following MRK-3 treatment (Sankaranarayanan et al., 2009), sAPPα concentration in CM was measured using a combination of 6E10 capture antibody (Aβ aa 3–16) and detecting antibody P2-1-AP (recognized APP sequence aa104–118) similar to that previously described (Sankaranarayanan et al., 2008; Wu et al., 2011). To confirm the specificity of the NFEV ELISA assay for Aβ peptides in the conditioned media, CM was first immunodepleted with streptavidin beads coated with biotin-labeled control mouse IgG, or 6E10, or 4G8 overnight. The EV_Aβ peptide concentration in the supernatant before (neat CM) and after immunodepletion was measured as described above.

Measurement of brain EV_Aβ peptides in APP_NFEV knock-in transgenic mice

The generation and characterization of APP_NFEV mouse model in which the endogenous BACE cleavage site "KMDA" was replaced with the "NFEV" sequence has been previously described (Simon et al., 2004; McCampbell et al., 2011). Homozygous and heterozygous APP_NFEV knock-in mice were identified by genotyping as previously described (McCampbell et al., 2011). Brain samples from these mice were extracted using 10 × (*w/v*) 0.2% DEA buffer and neutralized with 10% volume of pH 6.8, 0.5 mol/L Tris buffer (Sankaranarayanan et al., 2008). Brain Aβ_EV40 and Aβ_EV42 were measured using an ELISA similar to that described previously (Sankaranarayanan et al., 2008; Wu et al., 2011). Briefly, 96 microtiter well plates were coated with 2 µg/mL EV3 IgG antibody overnight, then washed once with PBST and blocked with 3% BSA/PBS. Aβ_EV40 or Aβ_EV42 standard peptides or diluted NFEV mouse brain homogenates (100 µL) were added followed by either Aβ40 or Aβ42 specific C-terminal neoepitope monoclonal antibodies conjugated with alkaline phosphatase (Sankaranarayanan et al., 2008) and incubated overnight at 4°C with shaking, Next day, the plates were washed with PBS, 0.05% tween and developed as described above. The concentrations of brain Aβ_EV40 and Aβ_EV42 were calculated using a third order spline fit to the standards and coeffi cients derived from respective standard curves.

ABBREVIATIONS

Aβ, amyloid-β; AD, Alzheimer's disease; APP, amyloid-β precursor protein; EC_{50}, half maximal effective concentration; ELISA, enzyme-linked immunosorbent assay; FRET, fluorescence resonance energy transfer; HPLC, high-performance liquid chromatography; IC_{50}, half maximal inhibitory concentration; MS, mass-spectrometry

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