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Identification of Novel Small Molecule Inhibitors of APP Protein Synthesis as a Route to Lower Alzheimer's Disease Amyloid-β Peptide

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Abbreviations: Aβ, amyloid beta peptide; AChE, acetylcholinesterase; AD, Alzheimer's disease; ADDL, Aβ derived diffusible ligands; APP, amyloid precursor protein; LDH, lactate dehydrogenase; LTP, long-term potentiation; UTR, untranslated region.

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Abstract

A wealth of independent research with transgenic mice, antibodies and vaccines has pointed to a causative role of the amyloid-β peptide (Aβ) in Alzheimer's disease (AD). Based on these and earlier associative studies, Aβ represents a promising target for development of therapeutics focused on AD disease progression. Interestingly, a cholinesterase inhibitor currently in clinical trials, phenserine, has been shown to inhibit production of both amyloid precursor protein (APP) and Aβ. We have shown that this inhibition occurs at the post-transcriptional level with a specific blocking of the synthesis of APP relative to total protein synthesis (Shaw et al., 1999). However, the dose of phenserine necessary to block APP production is far higher than that needed to elicit its anticholinesterase activity and it is these latter actions that are dose limiting in vivo. The focus of this study was to screen 144 analogues of phenserine to identify additional small molecules that inhibit APP protein synthesis and, thereby, Aβ production, without possessing potent acetylcholinesterase (AChE) inhibitory activity. An ELISA assay was used to identify analogues capable of suppressing APP production following treatment of human neuroblastoma cells with 20 μM of compound. Eight analogues were capable of dose-dependently reducing APP and Aβ production without causing cell toxicity in further studies. Several of these had little to no AChE activities. Translation of APP and Aβ actions to mice was demonstrated with one agent. They thus represent interesting lead molecules for assessment in animal models, to define their tolerance and utility as potential AD therapeutics.

Introduction

Alzheimer's disease (AD) is characterized by the progressive loss of cognition and the appearance of extracellular plaques and intracellular neurofibrillary tangles. While the exact cause of AD remains to be fully elucidated, evidence implicating the amyloid-β peptide (Aβ), a key component of extracellular plaques, is growing. For example, monoclonal antibodies and vaccines directed against Aβ have led to the reduction of plaques in transgenic mice (Chauhan, 2003; Lombardo et al., 2003) and non-human primates (Lemere et al., 2004). More importantly, immunization has led to improvements in cognition in transgenic animals (Younkin, 2001). Although a clinical trial using a vaccine directed against Aβ was halted, pathological data from patients in the trial support the notion that Aβ clearance has a broader role in disease progression, including influencing tau phosphorylation (Nicoll, 2003). The impact of Aβ on tau phosphorylation has also been supported by immunization of triple-transgenic mice containing the Aβ, tau and presenelin 1 genes. In these mice, a decline in LTP occurred prior to the appearance of the plaques or tangles, and correlated with the accumulation of intraneuronal Aβ (reviewed in (LaFerla and Oddo, 2005)). Combined, these studies point to the importance of Aβ in learning and memory, suggest a causative role of Aβ in AD pathophysiology, and thus support it as a target for AD drug development and treatment.

An alternative approach to lowering Aβ by increasing its clearance, is to lower its initial formation. Acetylcholinesterase (AChE) inhibitors have been shown to effect amyloid precursor protein (APP) processing in cell culture, resulting in lower levels of Aβ, and thus may also have actions on disease course (Pakaski, 2003; Racchi et al., 2004). There are currently five drugs approved for the treatment of AD, four of which, Tacrine (Cognex®), donepezil (Aricept®),

rivastigmine (Exelon®) and galantamine (Reminyl®) are AChE inhibitors, to which the NMDA-receptor antagonist, memantine (Namenda®), has been recently added. AChE inhibitors are considered to be symptomatic drugs that, by inhibiting the activity of AChE, elevate acetylcholine (ACh) levels in brain by reducing its rate of hydrolysis. This important neurotransmitter stimulates the cholinergic system and thereby augments learning and memory. Whether or not actions on APP and Aβ translate from cell culture to *in vivo* and AD remains unknown.

Phenserine, a physostigmine analogue, is an experimental AChE inhibitor (Greig et al., 1995 and 2005a), that has reached clinical trails for AD. Besides its anticholinesterase action, it inhibits the increase in APP in rats following a nucleus basalis lesion (Haroutunian, 1997), a model that mimics the forebrain cholinergic loss of AD. Later studies (Shaw et al., 2001), showed that phenserine reduces APP production in cultured neuronal cell lines post-transcriptionally at the level of the 5'-untranslated region (UTR) of APP mRNA, leading to lower Aβ levels. This 5'-UTR had previously been demonstrated to contain a translational enhancer (Rogers et al., 1999; Brun et al., 2003) that includes both an iron-responsive element as well as an interleukin-response element (Rogers et al., 2002a). Using the 5' UTR in a reporter gene assay, a screen of FDA approved drugs resulted in sixteen molecules capable of reducing the expression of the reporter gene and lowering APP levels (Rogers et al., 2002b; Morse, 2004), with phenserine likewise demonstrating activity in this assay (Morse, 2004). Whereas phenserine did induce a transient increase in ERK levels and phosphorylated ERK, its action in lowering APP was not dependent of either as selective inhibitors of each failed to prevent phenserine's ability to reduce APP levels (Shaw et al., 2001). Furthermore, this action was non-cholinergically mediated as the

agent's AChE-inert chiral isomer, (+)-phenserine, similarly lowered APP (Shaw et al., 2001; Greig et al., 2005a).

The physiological function(s) of the various APP forms and proteolytic products remain a focus of research and some possess likely roles in tasks as diverse as synaptic plasticity and apoptosis (Morgan C, 2004; Reinhard et al., 2005; Sambamurti 2006). Nevertheless, the strategy of reducing Aβ via its precursor with phenserine, has not been associated with adverse effects in chronically dosed rodents and dogs in preclinical studies (Greig et al., 2005a). While this dual action molecule represents an interesting alternative to currently approved anticholinesterases, AChE inhibitors are by nature dose-limiting which may prevent achievement of the necessary concentration for optimally inhibiting Aβ production in humans. Accordingly, we investigated whether analogues of phenserine that lacked potent AChE inhibitory activity could be identified that would still allow reduction of Aβ levels. We have identified a number of such analogues, including the (+)-enantiomer of phenserine, posiphen, described previously (Shaw et al., 2001).

Methods

General Analogue Synthesis

A total of 144 analogues were synthesized and assessed. All were based one the hexahydropyrrolo[2, 3b]indole backbone of phenserine and included synthetic intermediates and analogues (for review Greig et al., 1995 and 2005a). All agents were greater than 99.9% chemically and chiral pure. General substitutions are shown in Figure 1.

Cholinesterase Activity

AChE and butyrylcholinesterase (BuChE) inhibitory activity was determined over a range of 0.3–10,000 nM against freshly prepared human erythrocyte AChE and plasma BuChE by the Ellman technique. The concentrations of analogues required to inhibit 50% enzymatic activity (IC_{50}) were determined in triplicate.

Cell Culture

Human neuroblastoma cell lines SH-SY-5Y and SK-N-SH were obtained from the American Type Culture Collection, culture medium was from Mediatech, Inc. (Herndon, VA) and FCS from HyClone (Logan, UT).

Initial Compound Screen

For initial screening, SH-SY-5Y cells were cultured in 96 well plates at $1x10^5$ cells/ml in 100 µl of DMEM/0.5% heat inactivated FCS containing either duplicate dilutions of analogues (final concentration of 20µM in 0.1% DMSO) or vehicle controls. The cells were incubated for 16 hr at 37^{o}C and 5% CO_2. A 75 µl sample of supernatant per well was collected in tubes containing RIPA buffer with 10x protease inhibitors (Roche) for soluble APP and LDH measurements. Cellular viability was additionally assessed by the addition of MTS reagent (Promega Corporation, Madison, WI) to the remaining cells.

Levels of APP produced by cells, in the presence and absence of analogues, were quantified by ELISA. APP 44-100 antibody (BioSource) was diluted to 2 µg/ml and absorbed to a Maxisorp plate overnight at 4^o C. After washing with ELISA buffer, 80 µl of media and 10 µl of supernatant were added to the wells and incubated overnight at 4^o C. 6E10-biotin antibody

(Signet Pathology) was added to each well and incubated at room temperature for 2 hr. The plates were washed three times and detection antibody was added for 30 min. Following three additional washes, TMB substrate (Moss, Inc.) was added and developed for approximately 20 min. The reaction was stopped with 0.18 M sulfuric acid and the plate was read at 450 nm on a Wallac Victor2 plate Reader. The background values from the non-cultured media control were subtracted from the sample values and secreted APP levels were expressed as % vehicle control. LDH activity was analyzed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). MTS activity was determined using the CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega).

Western Analysis of APP Levels

SK-N-SH cells were then analyzed for the ability of 8 analogues identified in the initial screen to inhibit APP protein production. Cells were plated into 24-well plates at a density of 2 X 10^5 cells/well in 500 µl of MEME/10% heat-inactivated FCS containing 50 ng/ml of recombinant human β-NGF (R&D Systems Inc., Minneapolis, MN). They were incubated at 37^{o}C and 5% CO_2 for 2 days; thereafter, FCS was reduced to 0.5% and, 24 hr later, analogues (A – H) were added in similar medium, in triplicate alongside vehicle controls. Supernatant was removed from the cells after 24 hr treatment, protein levels were determined and 15 µg of sample was mixed with Laemmli buffer, boiled for five minutes and loaded onto a 10% SDS-PAGE gel (Novex). The proteins were separated at 200 V for 45 min and transferred to nitrocellulose. Following blocking with 5% non-fat dry milk, the nitrocellulose was probed for 2 hours with 2.5 µg/ml of 22C11 anti-APP antibody (Boehringer Mannheim). After washing twice, anti-mouse IgG conjugated to horse radish peroxidase (Sigma) was added for 30 min, the filter was washed three

times and sample was detected by chemiluminesence. Cell viability was assessed by LDH measurement and MTS activity vs. controls, as described.

Aβ ELISA

Concentrations of secreted Aβ(1–40) produced by cells in the presence and absence of analogues were analyzed in supernatant samples by ELISA Colorimetric Kit, as per the manufacturer's instructions (BioSource International). Background values from the non-cultured media control were subtracted from the sample values and secreted Aβ levels were expressed as % vehicle control.

RNA Quantification

To assess whether or not reductions in APP protein levels were mediated at the transcriptional levels, SH-SY-5Y cells were incubated, as described, for 16 hr in increasing concentrations of analogues A - H. The maximal concentration of each, at which no adverse actions were observed, as assessed by LDH measurement and MTS activity vs. controls, was then chosen for analysis of RNA levels. RNA was isolated, and equal volumes were separated through a 1.2% agarose-formaldehyde gradient and probed for actin and APP using random primed ^{32}P-probes. Signals of APP and actin mRNAs were quantified by Phosphoimage analysis (Cyclone Packard Instruments)

Protein Synthesis

To determine if the changes in abundance of APP protein resulted from activity at the translational level, SH-SY-5Y cells were incubated in methionine and cysteine free DMEM for 1

hr following treatment (16 hr) with and without analogues A - H (at maximal concentrations), then pulsed with ^{35}S-labeled amino acids for 10 min in the presence of compound. The medium was carefully removed and the cells were re-suspended in lysis buffer (20 mM HEPES, 2 mM EGTA, 50 mM β-glycophosphate, 1 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol) containing protease inhibitors (PMSF, aprotinin, leupeptin and soybean trypsin inhibitor) and frozen at -80^{o} C until use. APP protein was immunoprecipitated overnight at 4^{o} using a polyclonal antibody ab2072 (Abcam) and protein A/G resin. The immunoprecipitated APP were eluted from the protein A/G resin with 30 µl of sample buffer containing 10% (v:v) b-mercaptoethanol. The immunoprecipitated protein was electrophoresed on a 10% tris-glycine gel, after which the gels were fixed and dried at 80^{o} C for 60 minutes. The dried gels were exposed to Phosphor Screen (Packard, Instrument Comoany, Inc., Meriden CT) overnight and and the APP signals were quantitated on a phosphor imager. The levels of newly synthesized APP were normalized by TCA precipitable counts.

Rodent Studies

Twenty-seven adult male C57Blk mice (Taconic Farms), mean weight 28 g, were administered either analogue E (2.5 or 7.5 mg/kg, i.p. once daily, N = 8 and 9, respectively) or vehicle (N = 10) for 21 consecutive days. Animals were killed within 90 to 120 min of the final dose, the brain was removed and right cerebral hemisphere frozen to –80^{o} C. Thereafter, hemispheres were homogenized and cell lysates containing whole protein were prepared according to manufacturer's instructions for enzymatic assay kits (R&D Systems, Inc). Briefly, brain samples were homogenized in 800 µl low salt 1 × extraction buffer (R&D kit) and incubated on ice for 10 minutes. The homogenates were then centrifuged at 10,000x g for 15 minute at 4° C. The

protein concentration in each sample was estimated according to biuret–derived assay (Pierce Biotechnology) and levels of APP and Aβ(1-40) were measured by Western immunoblotting and specific sandwich ELISA, respectively.

Results

Of the phenserine related analogues screened against human neuroblastoma SH-SY-5Y cells, 12 inhibited secreted APP, as determined by ELISA. Four of these compounds had previously been shown to have potent anti-cholinesterase activity and were thus not pursued further. The cholinergic action of the remaining agents is shown in Table 1. In comparison to phenserine (IC_{50} AChE 22 $\pm$ 2 nM; BChE 1560 $\pm$ 45 nM), all are significantly less potent against AChE ($p<0.05$, Dunnett's t test) and six were devoid of AChE activity. With respect to BChE inhibitory action, compounds ranged from potent (analogues C, E and H) to lacking activity. All possessed lipophilicity, as assessed by Clog P values (Table 1), that is commensurate with a high blood-brain barrier permeability. To ensure that actions on APP were not due to general cell toxicity, LDH and MTS assays were carried out. The two provided similar results and cellular viability, as assessed by the latter, is reported in Table 1. All 8 analogues were capable of reducing APP levels in a manner that could not be accounted for by cellular toxicity alone, as assessed by a ratio of APP/cellular toxicity (Table 1). The greatest inhibition of APP secretion observed following 24 hr treatment was between 60 - 70% (analogues A, B and E), although the latter two agents induced a 35 and 50% cell toxicity. For analogues A, C, D, F and H, APP reductions were achieved without loss of cell viability. There was no significant correlation between either AChE or BChE inhibitory activity and drug-induced reductions in sAPP or viability ($p>0.05$).

Compounds with an APP/toxicity ratio of less than 0.95 were assessed in greater detail in human neuroblastoma SK-N-SH cells that secrete detectable levels of Aβ.

While the ELISA assay allowed for rapid screening of the 144 phenserine analogues, to validate these results and ensure selectivity of the response, intracellular and extracellular APP levels were determined by Western analysis of SK-N-SH cells, at six concentrations (0.01 – 10 μM) for each of the eight described analogues, and are shown in Figure 2. The maximal chosen concentration (10 μM) had no effect on cell viability of SK-N-SH cells. In accord with the initial ELISA-based screening assay, all analogues lowered extracellular APP levels. This occurred does-dependently, with a maximal reduction of between 50 – 60% of control levels. Most analogues (B, C, E, G and H) decreased both the extra- and intracellular APP levels ($p<0.05$ vs. control, Dunnett's t test). A few analogues (A, D and F), decreased extracellular APP ($p<0.05$ vs. control, Dunnett's t test) without significant reductions of intracellular APP levels ($p>0.05$ vs. control, Dunnett's t test). Of particular interest, analogues A, B, C, E and H induced reductions in extracellular APP levels at sub micromolar concentrations ($p<0.05$ vs. control at ≤1 uM, Dunnett's t test).

A decrease in intracellular and extracellular APP in the absence of a corresponding decrease in APP mRNA, as assessed by Northern analysis (Figure 3), can be due to a change in posttranscriptional regulation through reductions in translational efficiency. To analyze translational effects, the levels of newly synthesized APP were determined by a brief, 10 min, incubation in the presence of radiolabeled amino acids, the APP protein was immunoprecipitated and the amount of label incorporated was normalized by TCA precipitable counts. The assay

was first optimized by titrating the antibody with a given amount of cell lysate. From this titration experiment, a saturation curve was established and a concentration of antibody was used for which total protein (300 μg) was at subsaturating levels. Using the optimized concentration of antibody, the APP signals were detected between 100 and 120 kDa and no other signals were observed indicating the specificity of the antibody:APP interaction. In addition, during optimization the compounds were shown to not interfere with antibody binding to APP. All analogues significantly decreased APP synthesis ($p<0.05$ vs. control, Dunnett's t test) (Figure 4) without changing TCA precipitable counts ($p>0.05$ vs. control), a measure of total protein synthesis. Of note, analogues B, C, D, E and H were some of the more effective compounds, inhibiting new APP protein synthesis by up to 60%.

The compounds were additionally evaluated for their ability to inhibit Aβ secretion in cultured media by treating cells at concentrations between 0.1 - 10 μM for 24 hr. As shown in Figure 2, which depicts the action of 0.3 - 10 μM dose vs. controls, several analogues (A, B, D, E and H) significantly lowered extracellular Aβ levels at 10 uM ($p<0.05$ vs. controls, Dunnett's t test). E and H were the most potent compounds, not only inhibiting Aβ accumulation in the media by up to 50% at 10 μM but also achieving a significant reduction at 1.0 μM ($p<0.05$ vs. controls, Dunnett's t test). In contrast, analogues A, C and F, although significantly lowering secreted APP levels by up to 60%, only mildly lowered Aβ levels by up to 20%.

Daily administration of analogue E to mice, as a representative of the eight APP lowering agents, was well tolerated. Neither dose (2.5 and 7.5 mg/kg, i.p. once daily x 21 days) affected body weight or induced any discernable aberrant or behavioral actions, compared to controls (not

shown). However, both doses significantly lowered brain APP (36 and 38%) and Aβ levels by 38 and 30%, respectively, vs. controls ($p<0.05$, Dunnett's t test vs. control) (Figure 5).

Discussion

The AChE inhibitor, phenserine, has previously been demonstrated to inhibit Aβ production at the posttranscriptional level, by inhibiting the synthesis of the APP protein (Shaw et al., 2001). Early work on the post-transcriptional regulation of APP had focused on elements in the 3' UTR involved in stabilizing the mRNA. Malter and colleagues demonstrated protein binding to a 3' UTR cis-element in the APP RNA. Furthermore, they demonstrated that hnRNP C and nucleolin interact with the 67 nucleotide element and enhance the stability of the mRNA by 6-fold (reviewed in (Malter, 2001)). Preventing the binding of hnRNP C to the APP mRNA should result in a destabilization of the mRNA and reduction in APP protein; however, hnRNP C is a ubiquitous protein found in most if not all cell types, and thus the direct targeting of this would likely have significant side effects.

The potential regulation of APP at the level of translation was first inferred from studies done by Wallace and colleagues (Wallace et al., 1995). They demonstrated that following a nucleus basalis lesion, there is a two fold increase in APP protein and the Aß peptide in the cortex of rats. Since an increase in protein stabilization would result in a decrease in proteolytic peptides, the increase in APP protein and Aß peptide must result from events occurring prior to protein synthesis. Additionally, they noted that this increase could be detected within one hour following the lesion. This eliminates transcriptional induction, which has been estimated to require 4 hours for the APP gene. Thus, the increase in APP protein is likely due to increased

translational efficiency of the mRNA. Additional studies demonstrated that this increase in APP could be eliminated by treatment of rats with phenserine (Haroutunian et al., 1997), being the first to raise the possibility that phenserine may be acting at the translational level to reduce APP synthesis.

The ability of phenserine to block translation was demonstrated by Greig and colleagues (Shaw et al., 2001). While one report (Mbongolo Mbella, 2000) identified an element in the 3' UTR of APP involved in regulating its translation, most of the studies support a role for the 5' UTR. Rogers first identified a 90-nt translational enhancer in the 5' UTR (Rogers et al., 1999) and later found that this region contained an iron-responsive element (Rogers et al., 2002a). Mallet and colleagues (Brun et al., 2003) also demonstrated the translational enhancer activity of the APP 5' UTR in neuronal cells when placed in front of a reporter gene. Utilizing this UTR in a reporter gene assay and screening a panel of FDA approved compounds, a number of inhibitors of APP translation were identified (Rogers et al., 2002b; Morse, 2004). Although many of these hits likely have restricted brain access consequent to their limited blood-brain barrier permeability or may exhibit toxicity with chronic use, there were four compounds that inhibit APP for which their primary mechanism of action is neuronal. Of these, phenserine appeared to be the most efficacious.

Whereas phenserine has also been shown to be capable of lowering Aβ levels *in vivo*, the potent AChE inhibitory activity of the compound (IC_{50} 22 nM) raises concern as to whether dosages needed to optimally lower APP and Aβ clinically, might limited by cholinergically mediated side effects (e.g., nausea). In neuronal cultures, significant APP activity is achieved at >1 μM (Shaw

et al., 2001). Thus, the focus of the present study was to identify analogues of phenserine that still inhibit APP synthesis but lack AChE potency. In contrast, selective BChE inhibition has not been associated with classical cholinergic overdrive (Greig et al., 2005b) and lack of this enzyme activity occurs naturally in humans, consequent to mutation, without adverse effects.

Following the screening of a number of phenserine analogues in human SH-SY-5Y neuroblastoma cells, eight lipophilic ones were identified that varyingly inhibit intracellular and extracellular APP levels and the Aβ peptide in culture media of human SK-N-SH cells at concentrations that were largely devoid of AChE activity. Whereas all the identified agents lowered secreted levels of APP in a concentration-dependent manner in cell culture, this did not always translate to reductions in cellular APP and secreted Aβ, suggesting a complex regulation. A change in secreted APP absent a corresponding change in intracellular APP may result from changes in the processing or secretion of APP, which will be investigated in future studies. However, most of the compounds did decrease both intracellular and extracellular APP levels with a corresponding decrease in secreted Aβ. This was achieved without altering APP mRNA levels and appears to translate *in vivo*, as assessed by lowered brain APP and Aβ in mice administered analogue E. One possible mechanism by which protein levels are reduced without a change in RNA is through translational regulation of protein synthesis. The inhibition of APP protein synthesis is supported by the data showing a reduction in newly synthesized APP protein without changes in total protein synthesis or cell viability, following analogue treatment.

Protein synthesis is a complex process involving ribosome assembly, initiation and elongation. While the efficiency of translation of mRNA can be regulated at any of these steps, initiation,

which includes the level and activity of initiation factors, is predominantly the major regulatory event. The efficiency of translation of individual mRNAs can vary by a 100-fold and are regulated in response to nutritional, hormonal, and developmental signals (Gingras et al., 1999; Rhoads, 1999).

At least eight proteins have been identified as targets for regulatory pathways affecting protein synthesis through cap-dependent translation (Rhoads, 1999). The 4E-BP (eIF4E Binding Protein) has generated a great deal of interest in drug discovery as it is regulated via phosphorylation. Unphosphorylated 4E-BP is bound to eIF4E, preventing it from initiating protein synthesis. Upon phosphorylation of 4E-BP, which is dependent on mTOR (mammalian target of rapamycin) (Jacinto E, 2003), eIF4E is released and can bind to eIF4G to initiate protein synthesis (Haghighat et al., 1995). Other enzymes important in this signal transduction process include: PI3K, PDK, PKB, and Akt (Huang and Houghton, 2003). Rapamycin, the compound that inhibits mTOR activity, and a number of its analogues are in preclinical and clinical development (Dancey, 2002) providing validation for the approach of targeting protein synthesis therapeutically. Rapamycin has also been shown to regulate LTP in hippocampal slices (Tang et al., 2002).

Synaptic plasticity is well understood to play a major role in learning and memory which is linked to LTP, a measurement of calcium influx and thus neuronal activity. In addition, synaptic dysfunction has been shown to be a better predictor of memory and cognitive decline than either AD plaques or tangles (DeKosky ST, 1990; Dickson et al., 1995; Sze CI, 1997). Many of the signal transduction pathways, such as ERK and 4E-BP phosphorylation, that are involved in the regulation of protein synthesis have also been demonstrated to have an important

role in synaptic plasticity (Kelleher et al., 2004; Kelleher, 2004). Although phenserine does not seem to regulate APP levels through either ERK or PI3K (Shaw et al., 2001), its ability or the ability of some of the recently identified analogues to alter APP protein synthesis through other pathways remains to be determined.

Protein translation clearly plays an important role in synaptic plasticity. Within the neurons there is a great deal of protein synthesis occurring in localized regions including the synaptic sites of dendrites (Steward and Schuman, 2001; Steward, 2003). Furthermore, Krichevsky and Kosik (Krichevsky, 2001) showed that many mRNAs accumulate in neuronal granuoles where they are translationally inactive but are readily moved to polysomes in response to depolarization. In contrast, depolarization decreased mRNA levels and protein synthesis of APP (Tabuchu, 2004). This localized synthesis allows neurons to respond promptly to changes in synaptic signaling, leading to an increase or a decrease in the synthesis of particular proteins, which are likely to be essential early mediators of synaptic plasticity and signaling. In the present study, APP protein synthesis was rapidly affected following analogue treatment, with reduced synthesis detected within ten minutes. Whether these phenserine analogues are affecting the same signaling pathways as depolarization remains to be determined. The identification of molecules that selectively affect translation of APP without impacting AChE, not only provides for a potential therapeutic targeting reduction of the Aβ peptide in AD patients with a reduced side effect profile, but should also allow for a better means of studying the pathways involved in APP protein synthesis.

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Footnotes:

* These authors contributed equally to this work.

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Figure Legends

Figure 1. General Structure of Phenserine and Analogues A – H. Analogue A: N-Dimethyl, N^1-benzyl serotonin; Analogue B: (-)-N^8-Benzyl phenserine; Analogue C: (-)-N^1-Benzyl, 4'-benzyloxy phenserine; Analogue D: (+)-N^1, N^8-Dibenzyl phenserine; Analogue E: (-)-N^1-Benzyl, N^8-nor, 4'-isopropyl phenserine; Analogue F: (+)-Eseroline; Analogue G: (-)-4'-Bromo phenserine; and, Analogue H: (-)-N^1-Phenethyl, 4'-isopropyl phenserine.

Figure 2. Effects of Phenserine Analogues, A – H, on Extra- and Intracellular APP and Extracellular Aβ Levels in Human SK-K-SH Neuroblastoma Cells in Culture. Mean (± SEM) levels of extracellular and intracellular APP levels were determined by Western analysis in SK-N-SH cells after 24 hr incubation in the presence and absence of six concentrations (0.01 – 10 μM) of analogues A – H. Extracellular APP levels were significantly reduced by all analogues (A ≥ 0.03 μM, B ≥ 0.3 μM, C ≥ 0.1 μM, D ≥ 1.0 μM, E ≥ 0.3 μM, F =10 μM, G ≥ 0.3 μM, H ≥ 0.1 μM; $p \leq 0.05$, Dunnetts t test). Intracellular APP levels were significantly reduced by analogues B (≥ 3.0 μM), D (10 μM), E (≥ 3.0 μM), G (≥ 3.0 μM) and H (≥ 3.0 μM) ($p \leq 0.05$, Dunnett's t test). Extracellular Aβ levels were significantly lowered by analogues A, B, D, E and H at 10 μM as well as by E and H at 1.0 μM ($p < 0.05$, Dunnett's t test).

Figure 3. Action of Phenserine Analogues A to H on APP mRNA Levels in Human SH-SY-5Y Neuroblastoma Cells, as Determined by Quantitative RT-PCR. The maximal dose at which no toxicity was observed (LDH and MTS assays) was determined for each analogue and used to treat SH-SY-5Y cells for 16 hours. Total intracellular and extracellular protein was

isolated, APP levels were determined by Western analysis and showed parallel changes to those illustrated in Figure 2. APP mRNA levels (mean ± SEM) were measured by quantitative RT-PCR and were unchanged compared to control ($p > 0.05$, Dunnett's t test).

Figure 4. Translational Regulation by Analogues (Rate of APP Synthesis) in Human SH-SY-5Y Neuroblastoma Cells. Translation was assessed by addition of radiolabeled amino acids for 10 min followed by immunoprecipitation of the newly synthesized APP protein. Effects on total protein synthesis were determined by measuring TCA precipitable counts. The APP levels were then normalized to the total TCA precipitable counts. All analogues significantly reduced newly synthesized APP levels (mean ± SEM), compared to controls ($p < 0.05$, Dunnett's t test) Total protein synthesis, assessed by TCA precipitable counts, was unaffected by analogues ($p>0.05$, Dunnett's t test).

Figure 5. Effect of Analogue E on brain levels of Aβ in Mice. Analogue E (2.5 and 7.5 mg/kg, i.p.) or saline were administered to C57Blk adult male mice once daily for 21 days, and Aβ(1-40) was determined by ELISA in the right cerebral hemisphere at 90 – 120 min after the final dose. Analogue E significantly lowered Aβ levels (mean ± SEM) by 38 and 30%, respectively, compared to controls ($p < 0.05$, Dunnett's t test).

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Table 1. Cholinesterase inhibitory activity, and Extracellular APP and Cell Viability as a Percent of Control Values

	sAPP ELISA (20 μM)	Viability MTT (20 μM)	Ratio APP/MTT (20 μM)	IC_{50} AChE (nM±SEM)	IC_{50} BChE (nM±SEM)	Clog P*
A	35%	94%	0.37	>10,000	2600 ± 135	1.51
B	38%	65%	0.58	110 ± 9	40 ± 9	3.96
C	63%	99%	0.64	>10,000	9 ± 1.3	6.02
D	75%	94%	0.80	>10,000	>10,000	6.19
E	29%	50%	0.58	>10,000	3 ± 0.5	4.56
F	88%	94%	0.94	>10,000	>10,000	1.54
G	68%	84%	0.72	650 ± 95	1100 ± 175	3.10
H	84%	99%	0.85	>10,000	4 ± 0.2	5.72

*Clog P value is a calculated assessment of lipophilicity (log octanol/water partition coefficient) determined at pH 7.0. (Pallas, CompuDrug International). A value of log 1.0 and greater is commensurate with a rapid blood-brain barrier penetrability. There was no significant correlation between either AChE or BChE IC_{50} values and percent inhibition of sAPP or cell viability ($p>0.05$ for all coefficients of linear regression analyses)

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Figure 1

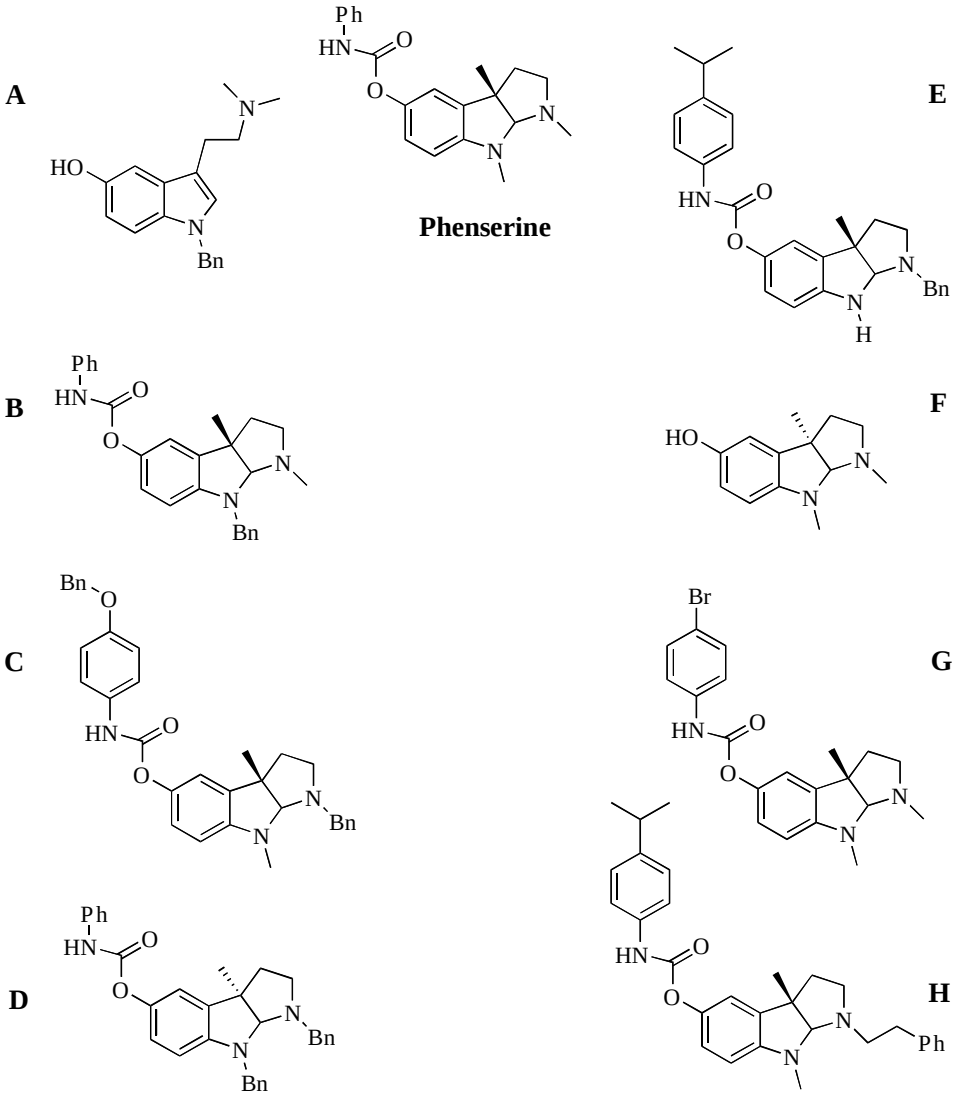


Figure 2

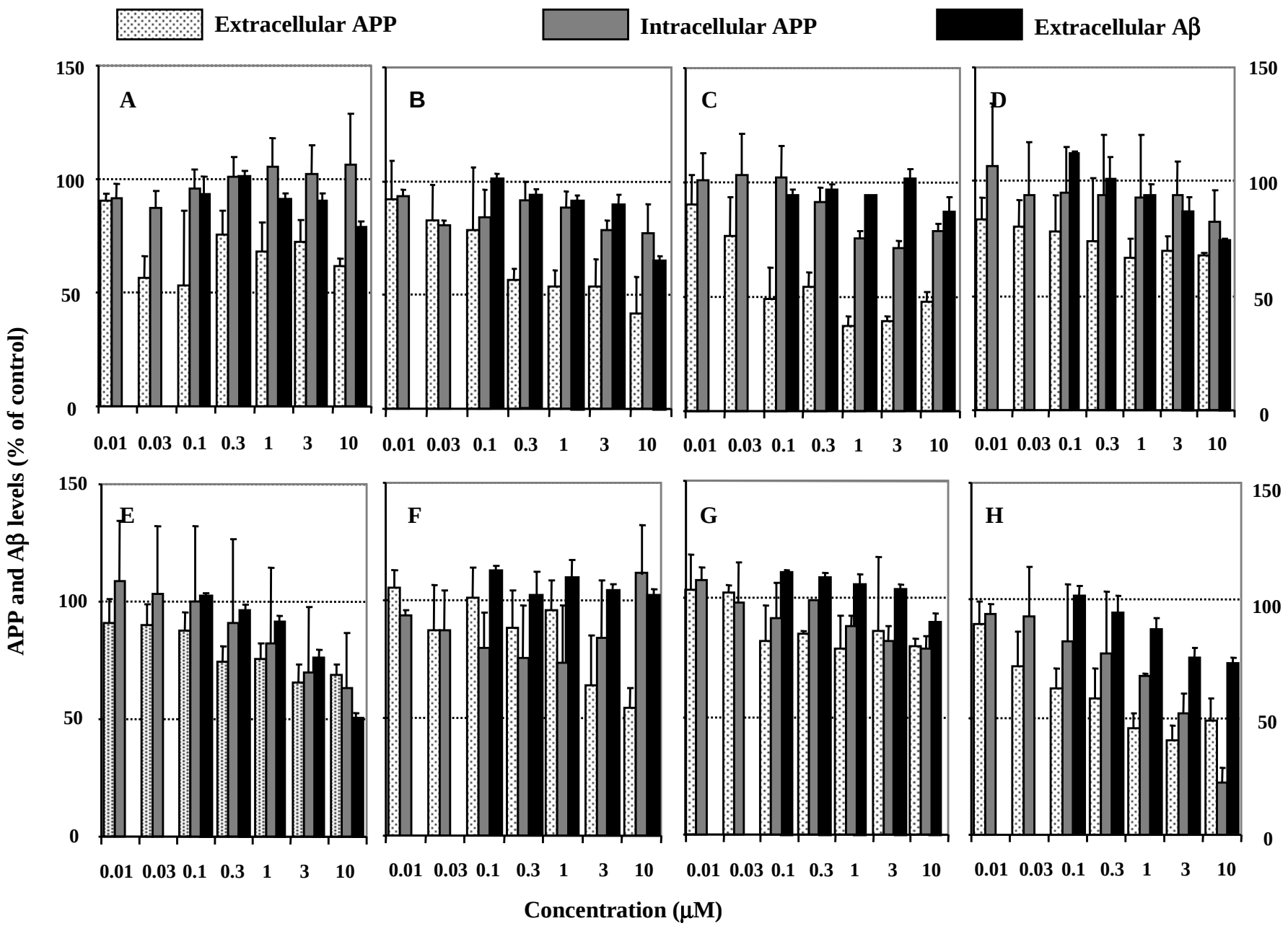


Figure 3

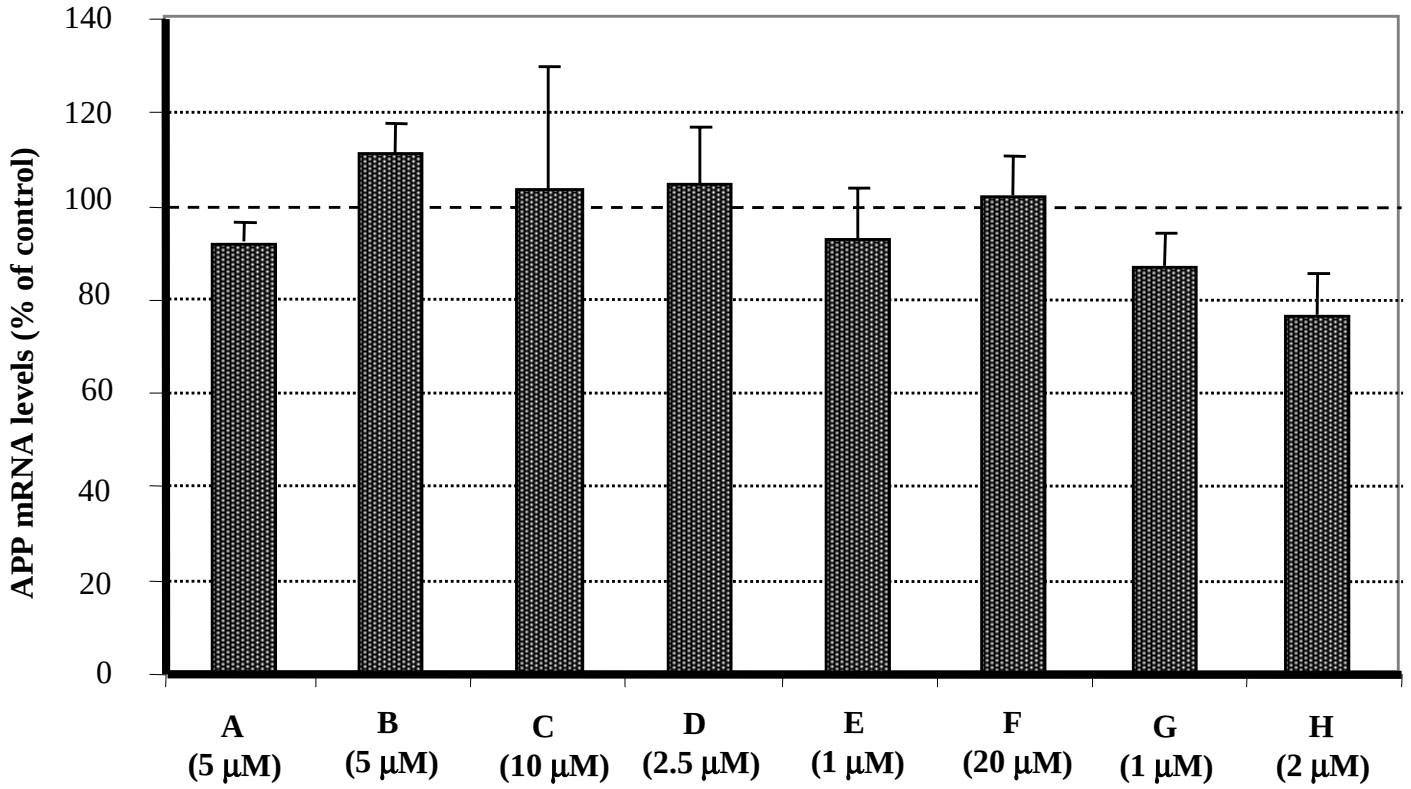
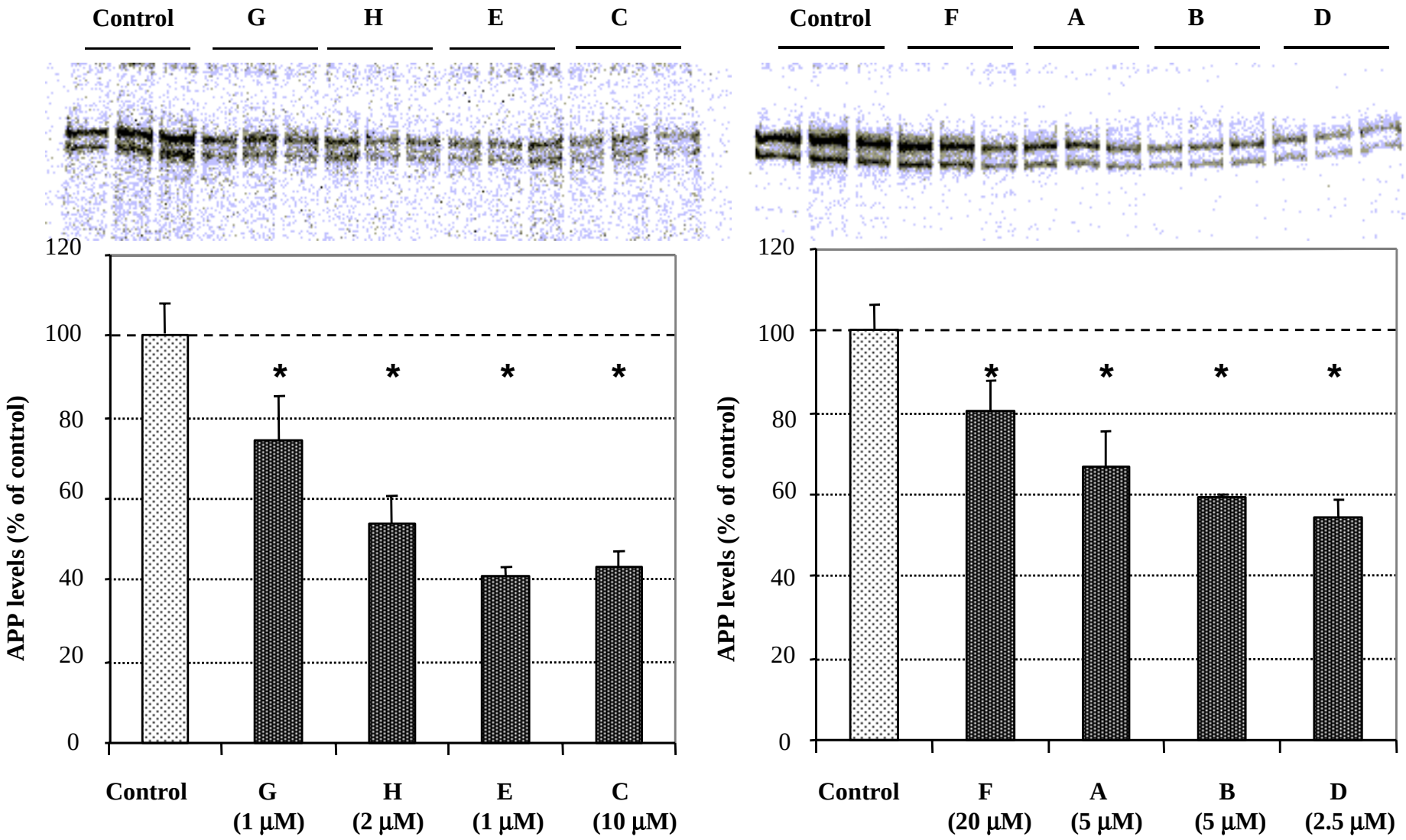


Figure 4



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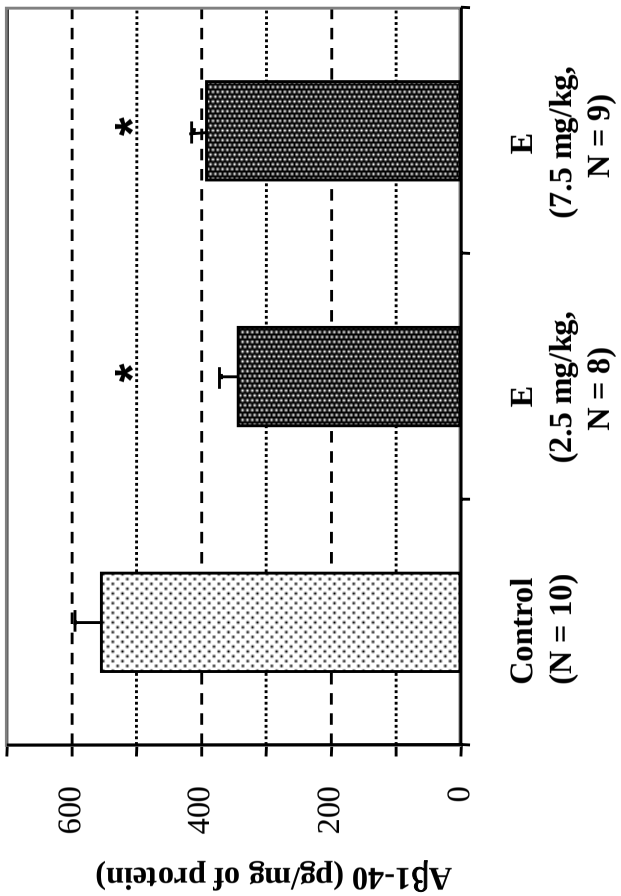

Figure 5