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A Novel Small Molecule Modulator of Amyloid Pathology

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Abstract

Because traditional approaches to drug development for Alzheimer's disease are becoming increasingly expensive and in many cases disappointingly unsuccessful, alternative approaches are required to shift the paradigm. Following leads from investigations of dihydropyridine calcium channel blockers, we observed unique properties from a class of functionalized naphthyridines and sought to develop these as novel therapeutics that minimize amyloid pathology without the adverse effects associated with current therapeutics. Our data show *methyl 2,4-dimethyl-5-oxo-5,6-dihydrobenzo[c][2,7]naphthyridine-1-carboxylate* (BNC-1) significantly decreases amyloid burden in a well-established mouse model of amyloid pathology through a unique mechanism mediated by Elk-1, a transcriptional repressor of presenilin-1. Additionally, BNC-1 treatment leads to increased levels of synaptophysin and synapsin, markers of synaptic integrity, but does not adversely impact presenilin-2 or processing of Notch-1, thus avoiding negative off target effects associated with pan-gamma secretase inhibition. Overall, our data show BNC-1 significantly decreases amyloid burden and improves markers of synaptic integrity in a well-established mouse model of amyloid deposition by promoting phosphorylation and activation of Elk-1, a transcriptional repressor of presenilin-1 but not presenilin-2. These data suggest BNC-1 might be a novel, disease-modifying therapeutic that will alter the pathogenesis of Alzheimer's disease.

Keywords

Amyloid- β protein precursor; Elk-1; presenilin-1; presenilin-2

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SUPPLEMENTARY MATERIAL

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INTRODUCTION

Alzheimer's disease (AD) is the sixth leading cause of death in the United States and today affects 5.2 million Americans aged 65 and over [1]. Without preventive strategies or development of an efficacious treatment, there may be 16 million Americans with AD by the year 2050 [2].

Currently the US FDA has approved five medications for treatment of symptoms of AD including Aricept (donepezil) approved for all stages of AD; Razadyne (galantamine) for mild to moderate AD; Namenda (memantine) for moderate to severe AD; Exelon (rivastigmine) for mild to moderate AD; and Cognex (tacrine, no longer commercially available in the US) for mild to moderate AD. More importantly, no new drugs have been approved since the FDA approved Namenda in 2003. Most current drugs (donepezil, galantamine, rivastigmine, and tacrine) are cholinesterase inhibitors aimed at slowing loss of acetylcholine, a key neurotransmitter that is significantly decreased in AD (reviewed in [3]). The other FDA approved drug, memantine, is an N-methyl-D-aspartate (NMDA) receptor antagonist that minimizes alterations in calcium flux associated with neuron degeneration in AD (reviewed in [4]). Because of the variability in patient response (reviewed in [3, 4]), there is a critical need for identification of additional disease-modifying therapeutics for patients early in disease progression that will alter the pathogenesis of the disease.

Pathologically, AD is characterized by an abundance of neurofibrillary tangles, senile plaques, neuropil thread formation, neuron and synapse loss; and proliferation of reactive astrocytes and microglia and the resulting neuroinflammation, particularly in the hippocampus, amygdala, entorhinal cortex, and neocortex. Neurofibrillary tangles are composed of intracellular deposits of paired helical filaments composed of hyperphosphorylated tau. Senile plaques are present as diffuse plaques composed of amorphous extracellular deposits of amyloid- β peptide ($A\beta$) that lack neurites; and neuritic plaques composed of extracellular deposits of insoluble $A\beta$ surrounded by dystrophic neurites, reactive astrocytes, and activated microglia. In addition to insoluble $A\beta$ present in senile plaques, soluble $A\beta$ oligomers are present in the AD brain and may represent the main toxic form of $A\beta$, thus implicating them in the disease process [5–7].

The current prevailing hypothesis for how AD develops involves the $A\beta$ peptide, which is produced from the larger amyloid- β protein precursor ($A\beta$ PP) through sequential cleavage by β - and γ -secretases. Cleavage of $A\beta$ PP by β -secretase (mostly β -site $A\beta$ PP cleaving enzyme-1 [BACE-1] in brain) leaves a membrane-bound C-terminal fragment that undergoes further cleavage by γ -secretase that consists of presenilin-1 (PS-1), nicastrin (NCT), presenilin enhancer-2 (Pen-2), and anterior pharynx defective 1 (Aph-1) [8] to form $A\beta$ peptides ranging from 37 ($A\beta_{37}$) to 43 ($A\beta_{43}$) residues with the majority 40 amino acids in length ($A\beta_{40}$). A small proportion of $A\beta$ (~10%) is 42 amino acids long ($A\beta_{42}$) and, due to the increased hydrophobicity and fibrillogenic nature of the peptide, it assembles into progressively higher order structures from dimers to insoluble amyloid (senile) plaques. As soluble assemblies of $A\beta$ accumulate, neurons and synapses degenerate [9] leading to progressive, irreversible memory loss and loss of activities of daily living. The amyloid hypothesis of AD is backed by substantial circumstantial evidence from multiple studies

[10] with the strongest evidence being genetic. Autosomal dominant mutations that cause early onset familial AD are present in A β PP or PS-1 (PS-2). PS-1 or PS-2 form the active site of γ -secretase which implicates the 2 AD causal mutations to either the substrate from which A β originates (A β PP) or the enzyme responsible for its final cleavage.

Because BACE-1 cleavage initiates A β PP processing, it has long been considered a prime therapeutic target. Although initial characterization of BACE-1 knock-out mice showed almost complete loss of A β levels in brain [11] without any undesirable phenotype [12], further study showed cognitive deficits [13–15], premature death [16], and hypomyelination [17, 18]. Initial pharmacologies against BACE-1 included peptidomimetic inhibitors that showed poor pharmacologic properties including poor blood-brain barrier penetration (reviewed in [19]). Second generation BACE-1 inhibitors demonstrated improved pharmacologic characteristics but were substrates for P-glycoprotein transporter mediated efflux [20, 21] which limited brain concentrations (reviewed in [19]). More recently, several third generation small molecule BACE-1 inhibitors have been developed that demonstrate satisfactory brain penetration and modulation of cerebral A β in animal models and are currently in early stage clinical trials with LY2886821 (Lilly), AZD3293 (AstraZeneca/Lilly), and MK-8931 (Merck) in Phase II or Phase II/III human trials. Although all three drugs showed no adverse effects in Phase I testing, Lilly's Phase II trial of LY2886821 was terminated because a small number of subjects developed abnormal liver biochemistries (reviewed in [19]). Merck's MK-8931 led to sustained dose-dependent decreases in cerebrospinal fluid A β in 32 mild to moderate AD patients and is currently in a Phase II/III combined clinical trial expected to conclude in 2017/2018. Interim safety analysis of 200 AD patients dosed for 3 months shows no apparent adverse effects (reviewed in [19]). Phase II/III trials of AZD3293 in 1,551 mild cognitive impairment and mild AD patients are currently in the planning stages. Although initial data suggest these small molecule BACE-1 inhibitors represent a potentially useful therapeutic for AD, they may ultimately be faced with similar difficulties that plagued γ -secretase inhibitors primarily because of the wide variety of substrates processed by BACE-1 (reviewed in [19]).

Additional therapeutics aimed at minimizing A β production have focused on modulation of γ -secretase activity. Early studies showed inhibition of γ -secretase diminishes A β formation, prevents A β aggregation, and reverses cognitive deficits in transgenic models of A β deposition [22]. Unfortunately, γ -secretase functions in the cleavage of multiple transmembrane proteins in addition to A β PP, in particular the Notch family of transmembrane receptors required for Notch signaling [23]. Subsequent studies showed prolonged dosing of pan γ -secretase inhibitors led to inhibition of Notch signaling likely through inhibition of PS-2 in addition to PS-1 and adverse changes in the GI tract, spleen, and thymus that likely limit the extent of A β inhibition [24–26]. Despite this potentially serious problem, γ -secretase inhibitors including LY-450139 (Semagacestat) (Eli Lilly; [27, 28]) and MK0752 (Merck; [29]) have been tested in humans. Unfortunately, clinical trials have been largely unsuccessful (reviewed in [30]). In particular, Phase III trials of Semagacestat were terminated before completion because of increased weight loss, appearance of skin cancers, and impaired immune function [31].

Pharmacologic approaches aimed at enhanced A β degradation have mainly focused on vaccinations. Initial active vaccination trials using Elan's AN1702 antibody against A β ₄₂ aggregates led to meningeal encephalopathy in 6% of the vaccinated patients and led to early termination of the studies [32]. More recent studies have focused on passive immunization with anti-A β antibodies. Bapineuzumab (Elan/Wyeth), a humanized monoclonal antibody (reviewed in [33]) that binds the N-terminal region of A β and is therefore unlikely to recognize monomeric or oligomeric A β , and solenuzumab (Eli Lilly), a humanized A β antibody that binds monomeric but not fibrillar A β recently failed to meet primary efficacy goals in mild to moderate AD patients, although pooled analyses suggest solenuzumab may provide cognitive benefits (reviewed in [34]). In addition, several studies have raised concerns that A β immunization might lead to the occurrence of small microscopic bleeds (microhemorrhages) characterized by Prussian blue staining and the presence of small granular hemosiderin particles in microglia associated with vasculature [35]. Although the mechanism by which microhemorrhages are produced is unclear, Wilcock et al. propose that antibody-A β complexes at the vasculature initiate an inflammatory response that leads to the activation of matrix metalloproteinases and the breakdown of the cerebrovascular tight junctions [36]. Based on evidence from animal models, more recent studies have evaluated whether microhemorrhages and/or vasogenic edema are present in human subjects provided active or passive immunization based on imaging abnormalities on T2* weighted fluid attenuated inversion recovery (FLAIR) MRI sequences (vasogenic edema) or T2* weighted gradient echo sequences (microhemorrhages with hemosideriosis) (reviewed in [33]). A recent blinded analysis of images from multiple clinical trials shows 17% of patients given bapineuzumab had increased edema [33] with 47.2% of edema positive and 4% of edema negative patients also showing microhemorrhages [33]. Because of these modest or negative outcomes, there is a profound need to identify alternative therapeutics that can modulate AD neuropathology without the adverse effects associated with current experimental treatments.

In recent studies we demonstrated that AD subjects taking calcium channel blocker anti-hypertensives showed a slowed progression to dementia and that nifedipine in particular was effective in the decrease of A β production through decreases in PS-1 [37]. During the course of our studies of nifedipine, we identified a functionalized naphthyridine byproduct of the parent compound that appeared to have comparable efficacy on the inhibition of A β production in *in vitro* models.

In the present study we describe the synthesis and testing of a novel brain permeant small molecule, methyl 2,4-dimethyl-5-oxo-5,6-dihydrobenzo[c][2,7] naphthyridine-1-carboxylate; (BNC-1; US Patent # 12/779345; pending), on A β ₄₂ production in *in vivo* and *in vitro* models of A β production and describe a potential mechanism by which protective effects occur.

MATERIALS AND METHODS

Synthesis and characterization of BNC-1

Synthesis of 2,6-dimethyl-4-(2-nitrosophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (compound 1)—Starting material (1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester; 50 mg, 0.145 mmol, Sigma-

Aldrich, St. Louis, MO) was dissolved in 20 mL acetonitrile in a Pyrex tube, sparged with helium, capped, and photolyzed with a 250 W halogen lamp (3M EVW) for 60 min. The solvent was removed by rotary evaporation and the product was obtained as a blue-green oil (45 mg, 94% yield). GC/MS analysis showed greater than 98.5% purity.

Synthesis of 2,4-dimethyl-5-oxo-5,6-dihydrobenzo[c][2,7]naphthyridine-1-carboxylic acid, methyl ester (BNC-1)—Compound 1 (40 mg, 0.122 mmol) was dissolved in 20 mL ethanol and mixed with glutathione (372 mg, 1.2 mmol, Sigma-Aldrich, St. Louis, MO) dissolved in 20 mL water and allowed to react at room temperature for 18 h. The reaction mixture was extracted twice with 50 mL ethyl acetate, the extracts combined and washed with 100 mL saturated NaCl solution and dried over anhydrous sodium sulfate. The ethyl acetate was removed by rotary evaporation and BNC-1 was obtained as a white solid (33 mg, 96% yield). GC/MS analysis indicated greater than 98% purity. Analytical characterization of BNC-1 was carried out using high-resolution mass spectrometry, carbon and proton NMR and X-ray crystallography and results are shown in the Supplementary Material.

Electrophysiology methods

To verify BNC-1 did not retain capacity as a calcium channel blocker, we examined its effects on voltage sensitive calcium channels (VSCC) as previously described [38–40]. Briefly, recording pipettes (glass capillary tubes) were pulled on a horizontal micropipette puller and fire polished immediately before recording. Tip resistance averaged 3 MΩ. Bath recording solution contained 111 mM NaCl, 5 mM BaCl₂, 5 mM CsCl, 2 mM MgCl₂, 10 mM glucose, 10 mM HEPES, 20 mM tetraethylammonium chloride (TEA), 0.01 mM 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), and 0.001 mM tetrodotoxin (TTX) (pH = 7.35, osmolarity = 300 mOsm). The pipette solution contained 145 mM methane sulfonic acid, 10 mM HEPES, 3 mM MgCl₂, 11 mM EGTA, 1 mM CaCl₂, 5 mM MgATP, 13 mM TEA, and 0.1 mM leupeptin (pH = 7.35, osmolarity = 290). All recordings were obtained using a Multiclamp 700B patch-clamp amplifier (Molecular Devices Corporation, Sunnyvale, CA). Data were filtered at 2 kHz and digitized at 5 kHz. Voltage commands and data acquisition were controlled by pCLAMP software. All experiments were conducted at room temperature.

Prior to recording, junction potentials were nulled in the bath using the pipette offset control on the Multiclamp 700B. Seal quality was determined using the seal test feature of pCLAMP, and other membrane and recording parameters such as membrane capacitance and resistance, access resistance, and holding current were calculated using the membrane test feature (with filter settings at 10 kHz). These parameters did not differ across drug treatment conditions and were similar to those previously reported [38–40]. After the whole-cell recording configuration was established, cell membrane potentials were maintained at –70 mV. *I/V* relationships for each cell were established by successively stepping the membrane potential in 10 mV increments from holding to +60 mV. Cells were then stepped to their maximal activation voltage a total of three times (30-s interstep interval) to establish baseline VSCC activity and were then perfused with recording medium containing either 10

μM nifedipine, 10 μM BNC-1, or 0.1% DMSO vehicle and maximal VSCC activity monitored for an additional 10 min.

In vitro model of A β ₄₂ production

In vitro studies of BNC-1 were carried out using H4 neuroglioma cultures stably transfected to overexpress a Swedish APP mutation (APP₆₉₅ NL) leading to overproduction and secretion of A β ₄₂ into the culture medium [41]. Cultures were maintained in Opti-minimum essential medium (Opti-MEM) supplemented with 10% (vol/vol) fetal bovine serum and 5 mg/ml hygromycin and were split 1:2 every two days. For experiments, cultures were plated at a density of 2.5×10^5 cells/well in 6-well culture plates (Nunc) in Opti-MEM supplemented with 10% FBS and were maintained for 24 h in a 37°C incubator (5% CO₂). For treatment, complete medium was replaced by Opti-MEM without serum after 3 washes in serum free medium. Dose response toxicity curves were established by treating cultures with BNC-1 from 100 nM to 5 μM and cell viability assessed by quantification of the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as a measure of mitochondrial viability as described by Mossman et al. [42].

A β ₄₂ quantification and western blot analysis of proteins involved in A β processing

For quantification of A β ₄₂ production and levels of proteins involved in A β processing, H4 cultures were plated at a density of 2.5×10^5 cells/well in 6 well culture plates. Based on cell survival assays, cultures were treated for 16 h with 2.5 μM BNC-1 in serum free Opti-MEM. After treatment, medium (750 μl) was collected from each well and added to tubes containing 1 μM EDTA and frozen at -80°C until used for A β quantification using A β ₄₂ ELISAs (Innogenetics) per manufacturer's instructions. After medium collection, cultures were washed 3 times with PBS and cells scraped into RIPA buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄ plus a complete protease inhibitor cocktail (Roche Life Science). Cells from one plate were combined to generate 1 sample with 4 to 6 plates (samples) per treatment generated over 2 to 3 experiments. The cells were incubated on ice for 30 min, homogenized for 30 s using pulsed sonication, and centrifuged at $14,000 \times g$ (4°C) for 30 min. The supernatant was carefully collected and the protein content determined using the Pierce BCA method per manufacturer's instructions. For western blot analysis of proteins, aliquots (25 μg) of protein were separated on 4–20% SDS-PAGE gels, transferred to nitrocellulose and probed with antibodies against proteins involved in A β processing including BACE-1 (R & D Systems; 1:1550 dilution; MW identified = 56 kDa), PS-1 (Cell Signaling; 1:750 dilution; MW = 55 kDa [full length]), NCT (Cell Signaling; 1:1500 dilution; MW = 120/110 kDa), APH-1 (GenScript; 1:1500 dilution; MW = 29 kDa), and ADAM-10 (presumed α -secretase; Santa Cruz; 1:1500 dilution; MW (active) = 60 kDa). Cleaved Notch-1 levels were also quantified (Abcam, MW = 95 kDa) to verify that BNC-1 does not impair Notch processing. Total A β PP was quantified using CT-20 antibody provided by Dr. M. Paul Murphy (University of Kentucky) as described [43].

Standard receptor inhibition screening of BNC-1 for any of the 45 standard dopaminergic, adrenergic, GABA, histamine, muscarinic, norepinephrine, δ -, κ -, μ -opioid, nonopioid

intracellular or peripheral benzodiazapine receptors was carried out by the NIMH Psychoactive Drug Screening Program (UNC, Chapel Hill) as previously described [44].

Treatment of APP/PS1 knock-in mice

Double homozygous knock-in mice expressing a Swedish familial APP mutation and a humanized A β sequence and the 246 L PS-1 mutation (APP/PS1) from Cephalon Inc. were used to determine potential protective effects of BNC-1. The APP/PS1 mouse has been extensively characterized and develops small numbers of amyloid deposits in an age-dependent manner beginning at ~9 months of age in a pattern similar to the age dependent accumulation of pathology in AD. All animal studies were carried out under University of Kentucky IACUC approved protocols. For initial characterization of effects of BNC-1 on A β processing, we provided 4 APP/PS1 mice (13 months old) 50 mg/kg/d BNC-1 or vehicle alone as a single dose by oral gavage for 7 days. For long term treatment with BNC-1, male APP/PS1 mice ($n = 10$) were provided a control diet or diet containing BNC-1 ($n = 10$) beginning at age 6 months continuing to 13 months of age to include the ages during which pronounced A β deposition occurs. A custom standard rodent diet containing 700 ppm BNC-1 was prepared and pelleted by Purina. Average daily consumption of diet showed the mice consumed 50 ± 5 mg/kg/d BNC-1.

Following treatment, spatial learning in mice treated from 6 to 13 months of age was assessed using the Morris water maze (MWM) [45] by the University of Kentucky Rodent Behavior Core (UK-RBC) under IACUC approved protocols. The MWM has been used extensively to test spatial learning in mouse models of AD pathology [46] and was used under standard conditions. In the task, a mouse was placed in a black plastic pool of water (22 ± 1 °C) virtually divided into 4 quadrants that contained a black 10 cm diameter escape platform hidden just beneath the surface of the water and surrounded by focally illuminated salient distal cues mounted on walls. Mice underwent five days of training by placing them in each of the four quadrants (north, south, east, and west) and allowing them to swim for a maximum of 60 s to find the submerged platform. Following the final training session on day 5, the platform was removed and the mice were tested in a probe trial. Performance was recorded using a computer-based video tracking system to monitor escape latency to previous platform location and the distance to reach the previous platform position during the probe trial. Previous studies indicate APP/PS1 mice show impaired performance in this test [47].

After behavioral testing, mice were euthanized by CO₂ asphyxiation per University of Kentucky IACUC approved protocols. Brains were quickly removed, the cerebellum removed, and the cortices bisected and flash frozen in liquid nitrogen. For the initial assessment of BNC-1 efficacy in mice treated for 7d, we chose to quantify different pools of a single A β species and chose to quantify levels of PBS-, SDS-, and formic acid (FA)-soluble A β ₄₂ as previously described [48] using commercially available ELISAs (Invitrogen). For mice subjected to long term treatment with BNC-1, we quantified both A β ₄₀ using in-house produced ELISAs as previously described [49], and A β ₄₂ using commercial ELISAs (Invitrogen) per manufacturer's instructions. Levels of proteins involved in A β processing were quantified in the same tissue specimens by subjecting

aliquots (25 µg) of protein from the crude PBS preparation to separation on 4–20% SDS-PAGE gels, transferring to nitrocellulose and probing with antibodies described above. In addition, levels of ADAM-17 (presumed α -secretase; Abcam; MW = 93 kDa), synaptophysin (Abcam; MW = 38 kDa), and synapsin (Santa Cruz; MW = 80 kDa) as markers of synaptic integrity and total A β PP (CT-20; M. Paul Murphy, University of Kentucky 1:1000) were also quantified in mice provided long term treatment with BNC-1. Band intensities were quantified using a Li-Cor system and results normalized to levels of GAPDH (Santa Cruz) or β -actin (Abcam) on each gel. Results are expressed as mean $\pm$ SD % vehicle staining. For immunohistochemical staining of A β deposition, one hemisphere from a subset of mice treated with control diet or 50 mg/kg/d BNC-1 from 6 to 13 mo was drop fixed in 5% paraformaldehyde for 7d. After paraformaldehyde fixation, the tissue was dissected into three segments and embedded in paraffin. Sections (5 µm) were cut using a Shandon Finesse microtome, placed on Plus slides, and immunostained for A β using a monoclonal antibody raised against A β _{17–34} (Vector Laboratories, Burlingame, CA) and standard protocols.

To verify BNC-1 targets PS-1 expression and is not simply a pan γ -secretase inhibitor, we carried out γ -secretase inhibition assays as previously described [50] using an internally quenched peptide substrate consisting of the C-terminal β -A β PP amino acid sequence cleaved by γ -secretase (NMA-Gly-Gly-Val-Val-Ile-Ala-Thr-Val-Lys (DNP)-D-Arg-D-Arg-D-Arg-NH₂; Calbiochem) and membrane fractions containing γ -secretase prepared from 5XFAD transgenic mice overexpressing three human APP and two PS1 FAD mutations.

Quantification of drug concentrations in tissue samples

Levels of BNC-1 were quantified in tissue using an in-house developed method. A 50 µL aliquot of serum or homogenized brain was diluted with 50 µL of a 500 mM aqueous solution of ammonium acetate, 50 µL of methanol containing internal standard (N-methylated BNC-1, 1.75µM), and mixed for 30 s. An additional 150 µL of methanol in 50 µL increments was added with mixing to precipitate proteins and to extract BNC-1. The samples were centrifuged at 17,000 $\times$ g for 5 min to pelletize proteins. Five micro-liters of the supernatant was analyzed by reversed phase (water:Acetonitrile) HPLC/MS using an 1100 HPLC (Agilent, Santa Clara, CA) equipped with a Polymer X (100 $\times$ 4 mm, 3 µm particle size) HPLC column (Phenomenex, Torrance, CA) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo-Scientific, Waltham, MA) fullscan acquisition (m/z 100–700) at 70,000 resolution.

Statistical analyses

Statistical analyses for *in vitro* and *in vivo* studies were carried out using analysis of variance (ANOVA) with Dunnett's *post hoc* test for individual differences and Excel software.

RESULTS

BNC-1 identification and chemical characterization

In our previous studies we showed that nifedipine, a dihydropyridine calcium channel blocker, significantly decreased A β _{1–42} production in an *in vitro* model [37]. In the course of those studies, we observed that nifedipine solutions became more effective after exposure to ambient light in the laboratory. Characterization of nifedipine solutions exposed to light led to the identification of methyl 2,4-dimethyl-5-oxo-5, 6-dihydrobenzo[c][2,7]naphthyridine-1-carboxylate; (BNC-1; Fig. 1). To determine if BNC-1 retained capacity as a calcium channel blocker we measured whole-cell VSCC waveforms in primary rat cortical neurons treated with vehicle (0.1% DMSO), BNC-1 (10 μ M) or nifedipine (10 μ M) ($n = 4–6$ per group). Figure 2a shows representative whole cell VSCC waveforms elicited in cortical neurons by following step depolarization from -70 to $+10$ mV before and 10 min after drug application and demonstrates BNC-1 had no effect on VSCC whereas nifedipine caused a significant ($p < 0.001$) decrease in response. Figures 2b and 2c show VSCC currents (% baseline) for the full time course of the experiment (Fig. 2b) and mean $\pm$ SD peak VSCC currents (% baseline) for the final 1.5 min of the experiment (Fig. 2c) and demonstrate BNC-1 has no effect on VSCC. Further chemical characterization of BNC-1 is shown in the Supplementary Material. BNC-1 (molecular weight = 282.3 g/mol) meets many of the attributes of a successful CNS drug as described by Pajouhesh and Lenz [51] including a pKa of 10.5, the presence of only 1 H-bond donor, 4 H-bond acceptors and 1 rotatable bond. Standard receptor inhibition screening of BNC-1 by the NIMH Psychoactive Drug Screening Program (PDSP; UNC, Chapel Hill) showed BNC-1 is not a ligand for any of the 45 standard dopaminergic, adrenergic, GABA, histamine, muscarinic, norepinephrine, δ -, κ -, μ -opioid, nonopoid, or benzodiazapine receptors.

BNC-1 diminishes A β ₄₂ production in vitro

Previous studies show the H4 neuroglioma cultures overexpressing the APP₆₉₅ NL familial mutation show no alterations in A β PP maturation, C-terminal fragment accumulation, or secreted A β PP production [41] and in our hands produce quantifiable levels of the more neurotoxic A β ₄₂ fragment of A β PP. Initial *in vitro* dose-response studies in H4 neuroglioma cultures with concentrations of BNC-1 up to 2.5 μ M did not cause significant cell death measured by MTT reduction ($<10\%$ vehicle) whereas structural variants of BNC-1 were more toxic. To test the effects of BNC-1 on A β ₄₂ secretion into the medium by H4 cells, cultures were treated for 16 h with the maximum dose of BNC-1 that demonstrated minimal toxicity (2.5 μ M) and A β ₄₂ secreted into the medium quantified using standard ELISAs. In our hands, we see more robust production of A β ₄₂ by the APP₆₉₅ NL H4 cells and chose to focus on those measures. Results of the analyses showed BNC-1 treatment led to significantly decreased A β ₄₂ production with no significant effect on full length A β PP (Table 1). To begin to examine the mechanism by which BNC-1 treatment minimized A β , we quantified levels of proteins involved in A β production and found that BNC-1 significantly decreased levels of PS-1 and nicastrin, two key components of the γ -secretase complex but did not significantly alter levels of total A β PP. In contrast to standard γ -secretase inhibitors, BNC-1 did not significantly alter levels of PS-2 or significantly impact Notch-1 cleavage (Table 1). To verify BNC-1 targets PS-1 expression and is not simply a

pan γ -secretase inhibitor subject to the same adverse effects as existing γ -secretase inhibitors, we carried out γ -secretase inhibition assays as previously described [50] using an internally quenched peptide substrate consisting of the C-terminal -A β PP amino acid sequence cleaved by γ -secretase (NMA-Gly-Gly-Val-Val-Ile-Ala-Thr-Val-Lys(DNP)-D-Arg-D-Arg-D-Arg-NH₂; Calbiochem) and membrane fractions containing γ -secretase prepared from 5XFAD transgenic mice overexpressing three human APP and two PS1 familial AD (FAD) mutations. Results of the assay showed BNC-1 did not significantly decrease γ -secretase activity up to 1 μ M concentrations, whereas treatment with 100 nM L-685,458 (Tocris) a well characterized potent and selective γ -secretase inhibitor led to 45% inhibition.

To begin to address a possible mechanism by which BNC-1 selectively decreased PS-1, we quantified levels of total and phosphorylated Elk-1, a PS-1 transcriptional repressor, and found the drug significantly increased levels of phosphorylated (activated) Elk-1 without altering total Elk-1 levels suggesting the specific decrease in PS-1 may be mediated by activation (phosphorylation) of Elk-1 (Table 1).

Behavioral and biochemical effects of BNC-1 in APP/PS1 mice

To test if BNC-1 was brain penetrant and if it led to similar effects *in vivo*, we provided four 13-month-old mice expressing mutant APP and PS1 (APP^{NLh/ NLh} $\times$ PS1^{P246 L/P246 L} (APP/PS1)) vehicle alone or 50 mg/kg/d BNC-1 for 7 days as a single dose by oral gavage. Following treatment, mice were euthanatized and levels of A β ₄₂ and proteins involved in A β production were quantified. Consistent with the hypothesis that BNC-1 minimizes A β formation, Fig. 3 shows short term treatment with BNC-1 significantly decreased levels of A β ₄₂ in the more soluble PBS fraction suggesting that the drug minimized new formation of A β . The decrease in A β ₄₂ correlated with decreased levels of PS-1 and NCT suggesting BNC-1 quickly inhibits proteins involved in A β production.

To test the efficacy of BNC-1 against development of A β pathology, we provided male APP/PS1 mice with control diet ($n = 10$) or diet containing BNC-1 ($n = 10$) beginning at age 6 continuing to 13 months of age to include the ages during which pronounced A β deposition occurs. Quantification of average diet consumption showed the mice received a dose of 50 ± 5 mg/kg/d. Quantification of levels of BNC-1 in brain of a small number of mice ($n = 4$) following 8 h dosing in diet with 50 mg/kg/d showed mean $\pm$ SD serum levels of BNC-1 = 2.0 ± 0.8 μ M serum and 0.78 ± 0.29 μ M brain (wet weight), which corresponds to $\sim 0.1\%$ of the dose consumed and is comparable to the neuroprotective concentration used *in vitro*. Although these data suggest the drug has poor GI transport, protective effects were observed at this low circulating dose.

Following treatment, spatial learning and memory were assessed by the University of Kentucky Rodent Behavior Core (RBC) using the MWM. Figure 4 shows the mean $\pm$ SD distance swam to the platform for each training day and shows BNC-1 treated mice generally showed improved performance compared to vehicle treated mice although there were no significant differences for any of the individual days. Following the final training session on day five, the submerged platform was removed and the mice were subjected to a probe trial by starting them in the northwest quadrant and measuring the distance and

latency time to reach the position where the platform had been. Results of the probe trial showed BNC-1 treatment led to significantly shorter mean $\pm$ SD distance traveled to reach the position where the platform had been submerged (13780.6 ± 1518.2 cm) compared to APP/PS1 mice provided vehicle alone ($n = 9$) (16140.9 ± 2965.3 cm) (Fig. 4b). There was no significant improvement in latency time to the platform for any of the training or probe trials.

Following euthanasia, levels of PBS-, SDS-, and FA-soluble A β_{40} and A β_{42} were quantified as previously described [48]. Immunohistochemical staining of A β was also carried out on representative animals. Figure 5 shows BNC-1 treatment led to a substantial decrease in A β immunostaining (Fig. 5b) compared to animals provided vehicle (Fig. 5a). Quantification of the individual pools of A β_{40} and A β_{42} (Fig. 6) showed a general decrease in A β levels with significant ($p < 0.05$) decreases in SDS-soluble A β_{40} and FA-soluble A β_{42} . FA-soluble A β_{40} was also decreased in BNC-1 treated mice although the difference was not statistically significant. In addition, levels of proteins involved in A β processing were quantified in aliquots of the crude PBS preparation using western blot analysis as described above. Figures 7 and 8 show representative western blots (Figs. 7a, 8a) and quantification of levels of proteins (Figs. 7b, 8b) involved in A β formation and show BNC-1 treatment significantly decreased levels of nicastrin and both the full length and the C-terminal fragment of PS-1, but did not significantly alter levels of full length A β PP, full length or C-terminal PS-2, cleaved Notch-1, BACE-1, or the presumed α -secretases ADAM-10 or ADAM-17. To determine a potential mechanism by which BNC-1 uniquely decreases PS-1 without impacting PS-2 or cleaved Notch-1, we tested whether drug treatment led to alterations in levels of phosphorylated Elk-1, which can specifically bind to the -10 region of the PS-1 promoter and function as a potent dose-dependent transcriptional repressor of PS-1 a transcriptional repressor of PS-1 [52]. Figures 8a and 8b show mice treated with BNC-1 demonstrated no significant differences in total Elk-1 but showed a significant 60% increase in levels of activated Elk-1 phosphorylated at Ser389. Previous studies [53] demonstrate that p-Elk-1 is translocated to the nucleus where it serves as a transcriptional repressor of PS-1. Additionally, Figs. 8a and 8b show BNC-1 led to significantly increased levels of synaptophysin and synapsin suggesting enhanced synaptic integrity consistent with improved performance in the MWM.

DISCUSSION

Because of the central role of APP and/or PS mutations in familial AD and the well characterized toxicity of soluble A β , considerable research effort has been aimed at development of therapeutic agents that modulate A β formation/clearance including development of BACE inhibitors and inhibitors of the γ -secretase complex or studies aimed at enhanced clearance of A β through immunization. Unfortunately, multiple clinical trials of mono-therapeutics aimed at modulation of A β production/clearance in patients with AD have resulted in modest, if not negative, outcomes largely due to unexpected adverse effects associated with the therapeutic approaches. Despite these disappointing results, there remains a need for development of novel therapeutics that can minimize A β formation and toxicity but without adverse effects.

In the present study we describe the development and preliminary characterization of a novel, brain permeant small molecule (BNC-1) that significantly minimizes A β ₄₀ and A β ₄₂ production in APP/PS1 mice, a well characterized mouse model of amyloid deposition that shows an age-dependent increase in both SDS- and FA-soluble A β ₄₂ beginning at 6 months of age with pronounced increases in both at 12 months of age. Short term treatment of APP/PS1 mice with BNC-1 led to small but significant decreases in PS-1 and nicastrin and a corresponding decrease in PBS-soluble A β ₄₂ which likely represents newly generated A β ₄₂ in contrast to SDS- and FA-soluble A β ₄₂ that is likely accumulated over a longer period of time than the 7-day short term treatment. Our data further demonstrate that long term treatment of APP/PS1 mice with BNC-1 during the age span in which significant A β ₄₂ accumulation occurs leads to significant decreases in total A β demonstrated by immunohistochemistry and in both SDS-soluble A β ₄₀ and FA-soluble A β ₄₂ consistent with a prolonged decrease of PS-1 and nicastrin. Although our MWM data from the probe trial show improved performance for the distance traveled to the position where the platform had been located, there were no significant differences for latency time to platform. Results of the training trials showed a general improvement for BNC-1 treated mice although there was considerable variability among the mice which is consistent with recent studies that suggest the MWM may not be as effective as other tests (novel object recognition and radial arm maze) at the detection of behavioral deficits in APP/PS1 mice [54]. We are planning to use the alternative behavioral tests to characterize animals currently on a diet containing BNC-1. Our data also show that the BNC-1 mediated decrease in A β is not due to pan inhibition of γ -secretase activity but is rather associated with a selective decrease in levels of PS-1 and NCT, key components of the γ -secretase complex. Additionally, our data demonstrate that the selective decrease in PS-1 but not PS-2 levels may be mediated by an increase in phosphorylated (activated) Elk-1, a transcriptional repressor of PS-1 but not PS-2. Consistent with the suggestion that γ -secretase complex containing PS-2 may be largely responsible for cleavage of Notch-1, whereas γ -secretase containing PS-1 may have a more pronounced role in A β PP cleavage and A β production, our data show that BNC-1 significantly decreases A β production without significant impairment of Notch-1 cleavage. Although the exact target of BNC-1 remains unclear, our data show that in addition to diminishing A β production, treatment with the compound leads to significant increases in both synaptophysin and synapsin, key markers of synaptic integrity and neuron viability and improves one measure of learning and memory in the MWM.

Although several therapeutic compounds have been developed and tested for use in AD, none to date have proved useful in the modulation of the pathogenesis of AD and the cognitive decline associated with disease progression thereby leading to the need to identify novel therapeutics and modulation of alternative pathways that might diminish the pathologic progression and cognitive loss associated with AD. One potential novel pathway modified by BNC-1 that impacts A β pathology involves Elk-1, a member of the ternary complex factor (TCF) subfamily of ETS (E-twenty-six) oncogene family of transcription factors that is known to function as a transcriptional repressor of presenilin-1 [53]. Elk-1 is characterized by an ETS DNA-binding domain in addition to a short protein interaction motif (B-box), which allows interaction with a second transcription factor STF [55–57]. In brain, Elk-1 is expressed in neurons but not glia of the cortex, hippocampus, striatum and

cerebellum [58, 59]. Although Elk-1 expression is restricted to the nucleus in non-neuronal cells, mature neurons demonstrate both cytosolic and nuclear expression [58, 60]. In the cytoplasm Elk-1 binds to mitochondrial permeability transition pore complex during apoptosis [61] and can interact with tubulin, although the physiological role of the interaction remains unclear [62]. Translocation of cytoplasmic Elk-1 to the nucleus is mediated by phosphorylation at Ser 383 and 389 leading to disruption of Elk-1 binding to micro-tubules and mitochondria and orientation toward transcriptional properties at serum response element promoter regions [53]. Elk-1 can be phosphorylated by ERK [63–67], c-Jun N-terminal protein kinase (JNK) [68–72], and p38 MAP kinases. *In vivo* studies demonstrate the phosphorylation of Ser383 of Elk-1 in nuclear, somatic, and dendritic compartments following electrical stimulation of the corticostriatal pathway [58, 59]. ERK-mediated Elk-1 phosphorylation is also observed in hippocampal neurons following induction of long-term potentiation (LTP) and during contextual fear conditioning [73]. Additional studies demonstrate ERK-dependent Ser383/389 phosphorylation of Elk-1 is associated with increased CREB phosphorylation and zif268 mRNA induction following high frequency tetanus of the perforant path [74] and induction of LTP. These studies also demonstrate that MEK blockage led to inhibition of Elk-1 and CREB phosphorylation, zif268 mRNA induction and LTP [74] suggesting a prominent role of Elk-1 activation in LTP. Other studies show aversive taste-learning experience leads to transient phosphorylation of ERK, JNK, and Elk-1 in the insular cortex and that microinfusion of a MEK inhibitor completely abrogates Elk-1 phosphorylation and partially impaired conditioned taste aversion [75]. Similar effects on long-term memory were observed in visual cortex where ERK and Elk-1 are transiently phosphorylated by visual stimulation following prolonged dark rearing [76]. One-trial avoidance learning is also associated with increased ERK, Elk-1, and CREB phosphorylation and c-Fos expression in the hippocampus [77] that could be blocked by microinfusion of NMDA receptor antagonists. In the nucleus, Elk-1 targets components of core gene regulation machinery, basal transcription complex components, spliceosome subunits, and ribosomal proteins suggesting a role of Elk-1 in a coordinated regulation of basal transcription machinery [78]. In addition, Elk-1 appears to also function via complexes with SRF. Using ChIP-chip analyses Boro et al. showed ~22% of Elk-1 binding regions co-bound SRF [78]. Transcriptional repression of Elk-1 occurs through calcineurin (PP2B) mediated dephosphorylation [79], or through recruitment of co-repressor complexes containing histone deacetylase activities, which leads to chromatin compaction at the level of the promoters [53]. Perhaps most relevant to AD, Elk-1 binds specifically to the –10 region of the PS-1 promoter and functions as a potent dose dependent transcriptional repressor of PS-1 [52]. Taken together these data suggest Elk-1 activation may play an important role in PS-1 expression and in learning and memory and therefore may be a potential therapeutic target for AD.

Overall, the current study suggests that BNC-1 is well tolerated and significantly diminishes A β production in a mouse model of amyloid pathology without the adverse effects associated with current γ -secretase inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Structure of 2,4-dimethyl-5-oxo-5,6-dihydrobenzo[c][2,7]naphthyridine-1-carboxylic acid, methyl ester (BNC-1).

Fig. 2.

a) Representative whole cell voltage sensitive calcium channel (VSCC) waveforms elicited in primary rat cortical neurons by step depolarization from -70 to $+10$ mV before (1) and 10 min after (2) application of treatment with vehicle, 10 μ M nifedipine, or 10 μ M BNC-1. b) This panel shows full time course VSCC values (% baseline) for neurons treated with vehicle, 10 μ M nifedipine, or 10 μ M BNC-1 and shows no effect of BNC-1 whereas nifedipine led to a pronounced decrease in VSCC response. c) This panel shows mean $\pm$ SD peak VSCC currents (% baseline) measured over the final 1.5 min of the experiment and demonstrate BNC-1 has no effect on VSCC. $n = 4-6$ cells per treatment.

Fig. 3. Seven day treatment of 13-month-old APP/PS1 mice with 50 mg/kg/d BNC-1 by oral gavage led to significant decreases in levels of PS-1, NCT, and PBS-soluble A β ₄₂ in addition to increased levels of ADAM-10 which minimizes A β formation.

Fig. 4. Mean $\pm$ SD distance to platform for APP/PS1 mice treated with vehicle or BNC-1 for 5 days of training (a). Results represent the average distance to platform for trials beginning in each quadrant (north, south, east, west). b) This panel shows the mean $\pm$ SD distance traveled to find the position where the hidden platform had been located in the probe trial carried out on the fifth day of training and shows a significant improvement in distance traveled when the animals were released in the northwest quadrant.

Fig. 5. Immunohistochemical staining of A β shows significant plaque pathology in APP/PS1 mice provided vehicle (a) with minimal immunostaining in mice provided BNC-1 (b).

Fig. 6.

Quantification of PBS-, SDS-, and FA-soluble pools of Aβ₁₋₄₀ and Aβ₄₂ in APP/PS1 mice provided control diet or diet containing 50 mg/kg/d BNC-1 from 6 to 13 months of age. BNC-1 treatment led to significant decreases in SDS-soluble Aβ₁₋₄₀ and FA-soluble Aβ₄₂.

Fig. 7.
a) Representative western blots of C-terminal PS-1, PS-2, and cleaved Notch-1 proteins. b) Quantification of mean ± SD % vehicle Aβ₄₂ species and immunostaining for PS-1, PS-2, and cleaved Notch-1. Band intensities for each protein were normalized against GAPDH for each gel. **p* < 0.05.

Fig. 8.

a) Representative western blots of total and phosphorylated Elk-1, full length A β PP, ADAM-10, ADAM-17, NCT, synaptophysin, and synapsin in control and BNC-1 treated APP/PS1 mice. b) Quantification of immunostaining of each band normalized to GAPDH for each gel. Results expressed as mean $\pm$ SD % control. * p < 0.05.

BNC-1 significantly decreases A β ₄₂ production through Elk-1 mediated decreased PS-1 expression without significant impacts on cell viability. *n* = 6–9 samples over 3 experiments.

Table 1

	Mean±SD	% Vehicle	Survival (MTT)	A β ₄₂	Full Length A β PP	PS-1	PS-2	Cleaved Notch-1	p-Elk-1	NCT	BACE-1
Vehicle			100±13.6	100±5.4	100±38.7	100±5.8	100±52.9	100±16.8	100±7.5	100±2.8	100±1.9
BNC-1			92.8±8.9	35.9±5.7 *	140.2±50.7	73.5±10.9 *	117.2±22.7	107.0±41.0	117.5±5.4 *	81.2±14.1 *	102.3±2.9

* *p* < 0.05