

## Preliminary studies of a novel bifunctional metal chelator targeting Alzheimer's amyloidogenesis

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### Abstract

A growing body of evidence indicates that dysregulation of cerebral biometals (Fe, Cu, Zn) and their interactions with APP and A $\beta$  amyloid may contribute to the Alzheimer's amyloid pathology, and thus metal chelation could be a rational therapeutic approach for interdicting AD pathogenesis. However, poor target specificity and consequential clinical safety of current metal-complexing agents have limited their widespread clinical use. To develop the next generation of metal chelators, we have designed and synthesized a new bifunctional molecule—XH1, based on a novel 'pharmacophore conjugation' concept. This lipophilic molecule has both amyloid-binding and metal-chelating moieties covalently connected by amide bonds. It achieved a putative binding geometry with A $\beta$ 1-40 peptide by the computational chemistry modeling and reduced Zn(II)-induced A $\beta$ 1-40 aggregation in vitro as determined by turbidometry. Moreover, our pilot data indicated that XH1 has no significant neurotoxicity at low micromolar concentrations and acute animal toxicity. XH1 specifically reduced APP protein expression in human SH-SY5Y neuroblastoma cells and attenuated cerebral A $\beta$  amyloid pathology in PS1/APP transgenic mice without inducing apparent toxicity and behavior disturbances. Collectively, these preliminary findings carry implication for XH1 being a BBB-permeable lead compound for AD therapeutics targeting Alzheimer's amyloidogenesis, although further studies are needed.

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### 1. Introduction

Approximately 4.5 million Americans are affected by the most common senile dementia—Alzheimer's disease (AD), and at least \$100 billion is spent a year on direct care for AD patients. The problem is worsening as life expectancy

continues to increase. By 2050, the number of AD patients is projected to reach 13.2 million in the United States alone if no cure or preventive measure for AD is found. Thus, there is an urgent need of effective treatments for AD as it has quickly become public health hazard (Huang et al., 2004).

Genetic, biochemical, and neuropathological data strongly suggest that A $\beta$  amyloidogenesis is central in AD pathogenesis (Selkoe, 2001). Considerable efforts have been expended on studying the basic biology of amyloid precursor protein (APP) and other AD-related proteins, identifying the pathways of A $\beta$  metabolism.

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However, the neurochemical factors that promote the age-dependent A $\beta$  amyloidosis have received much less attention.

Mounting evidence indicates that dyshomeostasis of cerebral biometals such as Fe, Cu, Zn, and APP/A $\beta$ /metal–redox interactions, may contribute to the neuropathology of AD. First, studies have confirmed that insoluble A $\beta$  amyloid plaques in post-mortem AD brain had abnormal enrichment of Cu, Fe, and Zn (Lovell et al., 1998). Conversely, metal chelators dissolved these proteinaceous deposits from post-mortem AD brain tissue and attenuated cerebral A $\beta$  amyloid burden in APP transgenic mouse models of AD (Cherny et al., 1999, 2001). Second, we and others have shown that metals promoted the *in vitro* A $\beta$  aggregation into tinctorial amyloid (Bush et al., 1994; Huang et al., 1997; Atwood et al., 1998). Additionally, we have demonstrated that redox-active Cu(II), and to a lesser extent, Fe(III), were reduced in the presence of A $\beta$  with concomitant production of reactive oxygen species (ROS)—hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxyl radical (OH $\cdot$ ) (Huang et al., 1999a,b). These A $\beta$ /metal–redox reactions, which were silenced by redox-inert Zn(II) (Cuajungco et al., 2000), but exacerbated by biological reducing agents (Opazo et al., 2002), have engendered A $\beta$  oligomerization *in vitro* mediated by dityrosine cross-linking (Atwood et al., 2004). The very A $\beta$ /metal redox interactions may also lead directly to the widespread oxidation damages observed in AD brains (Smith et al., 1996) and contribute to AD pathogenesis consequentially. Third, the 5'-untranslated region (5'UTR) of APP mRNA has a functional iron-response element (IRE) (Rogers et al., 2002a,b) which is consistent with biochemical evidence that APP is also a redox-active metalloprotein (Multhaup et al., 1996). Fourth, compared to age-matched controls, gene expression levels for metal regulatory proteins such as metallothionein III (MT-III) and metal regulatory factor-1 (MTF-1) decreased more than 4 fold in AD brain (Colangelo et al., 2002). Moreover, MT-III protein was reduced in AD brain (Uchida et al., 1991; Yu et al., 2001). Hence, the emergence of redox-active metals as key players in Alzheimer's amyloid pathogenesis strongly argues that metal-complexing agents should be investigated as possible disease-modifying agents for treating AD.

In fact, an early study indicated that sustained intramuscular administration of a potent iron chelator—desferrioxamine (DFO) slowed the clinical progression of AD dementia (Crapper McLachlan et al., 1991). Moreover, a mild cognitive improvement has been reported for a small-scale open study of 30 AD patients following a 3 week treatment regime with another metal-complexing agent—clioquinol (CQ) (Regland et al., 2001). Further, a recent pilot phase II trial of CQ with double-blind placebo control has shown that the CQ treatment arrested cognitive decline and lowered plasma A $\beta$ 1-42 levels in AD subjects (Ritchie et al., 2003).

Although metal chelation may be a promising therapeutic strategy for AD, a major problem associated with

widespread clinical use of metal-complexing agents is their poor target specificity and consequential clinical safety. The long-term use of these agents is likely to perturb the homeostasis of many biometals and normal physiological functions of essential metal-requiring biomolecules.

Thus, a new class of metal-complexing agents that specifically target amyloid is required. Based on a novel 'pharmacophore conjugation' concept, we have recently designed and synthesized a bifunctional molecule—XH1, which contains both amyloid-binding and metal-chelating moieties. Herein, we present results from both *in vitro* and *in vivo* pilot studies of XH1. Our preliminary data suggested that this bifunctional lipophilic XH1 molecule was not neurotoxic at low micromolar concentrations and was able to: (i) bind to A $\beta$ 1-40 peptide putatively; (ii) decrease Zn(II)-induced A $\beta$  aggregation *in vitro*; (iii) specifically reduce APP protein expression in human SH-SY5Y neuroblastoma cells; (iv) attenuate cerebral A $\beta$  amyloid pathology in PS1/APP transgenic mice without inducing apparent toxicity and behavior disturbances. These findings imply that XH1 may cross blood–brain barrier (BBB) and hold the potential of being an amyloid-targeting metal chelator for AD treatment.

## 2. Materials and methods

### 2.1. The synthesis of bifunctional compound—XH1 and its putative binding with A $\beta$ 1-40 peptide

#### 2.1.1. The chemical synthesis and characterization of XH1

The design concept of XH1 molecule, which is ((4-benzothiazol-2-yl-phenylcarbamoyl)-methyl)-{2-[(2-[(4-benzothiazol-2-yl-phenylcarbamoyl)methyl]-carboxymethyl-amino}-ethyl)-carboxymethyl-amino]-ethyl}-amino)-acetic acid, was based on the conjugation of known functional moieties or 'pharmacophores'. The XH1 molecule contains one metal-complexing moiety (MCM) and two identical amyloid-binding moieties (ABMs) covalently linked by amide bonds. Synthesis of XH1 was carried out according to a procedure adapted from previously published synthetic methods (Shi et al., 1996; Klunk et al., 2001). The detailed synthetic procedure for XH1 has been described in a patent application—USSN 60/441,719, 10/762,965, and PCT/US04/01,669 filed by the General Hospital Corporation. The molecular identity of XH1 was confirmed by <sup>1</sup>H-NMR and mass spectrometry (MS).

#### 2.1.2. Computational search for the putative A $\beta$ 1-40/XH1 binding geometry

To obtain putative binding geometry between XH1 and A $\beta$ 1-40 peptide, the NMR structure of A $\beta$ 1-40 in 40% (v/v) trifluoroethanol/water (Sticht et al., 1995) was downloaded from protein databank ([www.rcsb.org/pdb/](http://www.rcsb.org/pdb/)) and a ligand (XH1)/receptor (A $\beta$ 1-40) docking module within a software package-SYBYL 6.9.1 (Tripos, Inc.) was applied.

This computational chemistry approach introduced XH1 into the energy field of A $\beta$ 1-40 peptide molecule and searched for optimum binding geometry between them. The operational procedure was the following:

- (1) Drew the chemical structure of XH1 and minimized its energy;
- (2) Read the chemical structures of both A $\beta$ 1-40 and XH1 into docking module of SYBYL;
- (3) Computed the Gasteiger-Hückel atomic charges associated with both A $\beta$ 1-40 and XH1 molecules;
- (4) Docked XH1 into A $\beta$ 1-40:
  - (a) Generated the 3D box along the potential binding area within A $\beta$ 1-40 molecular conformation for the XH1. The putative binding area was determined based on steric and electrostatic interaction between XH1 and A $\beta$ 1-40 molecules.
  - (b) Moved the XH1 translationally and rotationally into the 3D box while keeping interaction energy between A $\beta$ 1-40 and XH1 low.
  - (c) Obtained the putative binding geometry between A $\beta$ 1-40 and XH1.

## 2.2. Effects of XH1 upon Zn(II)-induced A $\beta$ 1-40 aggregation *in vitro*

Human A $\beta$ 1-40 amyloid peptide was synthesized by W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University). The synthetic A $\beta$ 1-40 peptide was characterized and prepared as previously described (Huang et al., 1997). In brief, synthetic A $\beta$ 1-40 peptide was dissolved  $\approx 300 \mu\text{M}$  in Milli-Q water (Millipore Corporation) and centrifuged at  $10 \text{ min} \times 10,000 \text{ g}$ . The supernatants were collected for determination of peptide concentration as previously described (Huang et al., 1997).

Zn(II) stock solution—Zn(II)-histidine was prepared by mixing National Institute of Standards and Technology (NIST) standards with L-histidine at Zn(II)/histidine molar ratio of 1:6 in Dulbecco's PBS (Sigma; composition: CaCl<sub>2</sub> 1.19 mM, MgCl<sub>2</sub> 0.6 mM, KCl 2.7 mM, KH<sub>2</sub>PO<sub>4</sub> 1.4 mM, NaCl 137 mM, Na<sub>2</sub>HPO<sub>4</sub> 7.68 mM, 'PBS'). The Zn(II) stock solution was adjusted to pH  $7.40 \pm 0.05$  before use.

To evaluate effects of XH1 on the Zn(II)-induced A $\beta$  precipitation, A $\beta$ 1-40 (10  $\mu\text{M}$ ) was co-incubated Zn(II)-histidine (10  $\mu\text{M}$ ) in PBS, pH 7.4, with XH1 (25  $\mu\text{M}$ ) or a potent metal chelator—Diethylenetriaminepentaacetic acid (DTPA, 25  $\mu\text{M}$ ), respectively. DTPA is a potent metal-complexing agent with log *K* values: Fe(II) 16.2, Fe(III) 27.7, Cu(II) 21.2, Zn(II) 18.2 (NIST Standard Reference Database 46, version 8.0). The incubation was performed at 37 °C in a flat-bottom 96-well microtiter plate (Corning Costar Corporation), and turbidity readings at 400 nm absorbance were recorded after 1 h incubation using a SpectraMAX Plus microplate reader directed by Softmax PRO version 2.1.0 software (Molecular Devices Corporation). Automatic 30 s plate agitation mode was selected for

the plate reader to evenly suspend the aggregates in the wells before all readings.

## 2.3. Neurotoxicity studies of XH1 in primary rat neurons

To evaluate the neurotoxicity of XH1, E17 rat cortical primary neurons were obtained from pathogen-free female Sprague-Dawley rats (purchased from Taconic Farms, MA) after 17 d of gestation, and cultured as described by Brewer and Cotman (1989). In brief, the neurons were grown at 95% O<sub>2</sub>, 5% CO<sub>2</sub>, 85% humidity, 37 °C for 4 d in serum-free Neurobasal™ medium with B-27 supplement (Life Technologies, Inc.), 20  $\mu\text{M}$  L-glutamate, 100 units/mL penicillin, 0.1 g/mL streptomycin, and 2 mM L-glutamine. On the 5th day (treatment day), the medium was replaced with serum-free Neurobasal™ medium plus L-glutamine without B-27 supplement and then treated with XH1 (0, 1, 10  $\mu\text{M}$ ). Neuronal cell viability was assayed 48 h after XH1 treatment. Both LDH release assays and MTT assays were performed for quantitative assessment of cell death and cellular metabolic activity using commercial kits (Roche).

## 2.4. Effects of XH1 on APP protein expression

### 2.4.1. Human SH-SY5Y neuroblastoma cell culture

Certain metal chelators such as DFO, CQ, and dimer-captopropanol (DMP) have been reported to interact with APP mRNA 5'UTR and suppress APP protein expression at micromolar concentrations consequentially (Rogers et al., 2002a,b; Payton et al., 2003). To evaluate the effects of XH1 on endogenous APP protein expression, human SH-SY5Y neuroblastoma cells purchased from the ATCC were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (BioWhittaker™) and antibiotics. When the cells reached 40–50% confluences, they were exposed to XH1 at various concentrations in 100% DMSO (0, 1, 5, 10  $\mu\text{M}$ ) under the same cell culture conditions (95% O<sub>2</sub>, 5% CO<sub>2</sub>, 85% humidity, 37 °C) for 48 h. To ensure the potential effects of XH1 upon APP protein expression are not due to treatment-induced cell death, the cell viability was also assessed after XH1 treatment using the LDH assay kit as described before.

### 2.4.2. SDS-PAGE analysis of XH1 effects on APP protein expression

Human SH-SY5Y neuroblastoma cells were collected and washed with cold PBS 3 times and then lysed with M-PER™ mammalian protein extraction reagent (Pierce) supplemented with protease inhibitor cocktails (Roche). The cell lysates were pelleted at 13,000 rpm  $\times$  15 min in a cold room, and the supernatants were BCA assayed for total protein concentrations (Pierce). Western blotting was performed on pre-casted NuPAGE™ 4–12% Bis-Tris gels (Invitrogen) with equal total protein loading (10  $\mu\text{g}$ /well). They were run at 200 V  $\times$  45 min and transferred to PVDF

membranes at 75 mA/gel $\times$ 90 min. The primary antibody was C-terminal rabbit polyclonal antibody for APP (A8717, Sigma). The blots were incubated at 1:1000 dilution (in TBST with 10% milk) overnight in the cold room, on a shaker. The blots were then washed for 1–2 h at 15 min intervals. For detection, anti-rabbit antibody at 1:10,000 was added to the blots for 45 min incubation at room temperature on a shaker. The blots were washed for 2–3 h at 30 min intervals, and then developed using a LumiGLO<sup>®</sup> chemiluminescence kit (KPL, Inc.). The final images were captured and analyzed by a VersaDoc<sup>™</sup> Digital Imaging System (BIO-RAD). Control proteins ( $\beta$ -tubulin and two amyloid-precursor-like proteins, APLP1 and APLP2) were also probed to ensure target specificity of XH1 towards APP. The primary antibodies for control proteins were: anti- $\beta$ -tubulin (T4026, Sigma), anti-APLP1 (Cat#171615, CALBIOCHEM), and anti-APLP2 (Cat#171616, CALBIOCHEM).

## 2.5. Pilot in vivo studies of XH1 using PS1/APP transgenic mice

### 2.5.1. Treatment of PS1/APP transgenic mice by XH1 and mouse brain tissue preparation

PS1(M146V)xAPPTg2576 (PS1/APP) transgenic female mice, ~6 month old, were used for the pilot in vivo studies. The mice were genotyped for both APP and PS1 transgenes before the experiments. The mice were treated with regular (3 mice) and XH1-formulated animal diets (5 mice) ordered from Purina Test Diets, Richmond, IN, USA, for 4 week. The mice on XH1-formulated diets received approximately 25 mg XH1/kg daily dose. The transgenic mice were closely monitored for general well being, food consumption rate, and gastrointestinal side effects. No apparent toxicity and behavior disturbances were observed during the treatment period.

Mice were deeply anesthetized and transcardially perfused with PBS, a whole brain was then dissected out from each mouse. One half of brain was used for immunohistochemical studies, and the other half was reserved for biochemical analyses.

### 2.5.2. Immunohistochemical staining for cerebral A $\beta$ amyloid in PS1/APP transgenic mice

The half brain tissues used for immunohistochemical staining were post-fixed with the Periodate–Lysine–Parafomaldehyde solution for 24 h, weighed, and cryoprotected in a graded series of 10 and 20% glycerol/2% DMSO solution. Brains were then serially cut at 50  $\mu$ m thickness on a freezing microtome and stained with cresyl violet to identify histopathological abnormalities. Sections were immunostained for A $\beta$ 1-42, A $\beta$ 1-40, GFAP and synaptophysin (SYP) as markers for A $\beta$  deposits, reactive astrocytes, and synapses. Immunohistochemical procedures were performed as previously described (Kowall et al., 2000). In brief, free-floating sections were incubated

overnight in primary antibody solutions followed by PBS washes and incubation in solutions of peroxidase-conjugated secondary antibody. The sections were developed using DAB as a chromagen. The following primary antibodies were used in immunostaining for brain sections of the transgenic mice: anti-A $\beta$ 1-42 (Cat#44-344, BioSource International), anti-A $\beta$ 1-40 (Cat#44-348, BioSource International), anti-GFAP (MAB3402, Chemicon), and anti-SYP (Cat#17750, Santa Cruz Biotechnology).

### 2.5.3. Cerebral A $\beta$ 1-40 measurements by ELISA

Sensorimotor cortex and striatum regions in the other half of each mouse brain were dissected out and placed in Eppendorf tubes and frozen in dry ice and stored in a –80 °C freezer for further experiments. Cold T-PER<sup>™</sup> tissue protein extraction solution (Pierce) supplemented with Complete<sup>™</sup> protease inhibitor cocktail (Roche) were added to the tubes by 500  $\mu$ L aliquots and homogenized thoroughly (1800 rpm) with a micro-tube pestle (Research Products International Corp.) mounted onto an electric motor (IKA Labor Technik) in the cold room. Homogenates were centrifuged at 13,000 rpm $\times$ 15 min in the cold room. Supernatants were carefully decanted, BCA-assayed for total protein concentrations (Pierce), and stored in a –20 °C freezer for further analyses. Colorimetric ELISA for A $\beta$ 1-40 in the supernatants was performed using a commercial kit (BioSource International).

## 3. Results

### 3.1. Bifunctional lipophilic XH1 achieved putative binding with A $\beta$ 1-40 computationally

As shown in Fig. 1A, the symmetric XH1 molecule has two identical amyloid-binding moieties (ABMs) and one metal-complexing moiety (MCM). The compound was characterized for its chemical identity by <sup>1</sup>H-NMR and MS. Its molecular weight (MW) was determined to be 809 Da, which was consistent with the chemical structure of XH1. The log *P* of XH1 was predicted by the ChemOffice<sup>®</sup> software suite (CambridgeSoft) to be 3.61, implying its high lipophilicity. In addition, a putative binding geometry between XH1 and A $\beta$ 1-40 (Fig. 1B) was accomplished by a computational ligand/receptor docking procedure using the software package—SYBYL. The binding geometry showed that the hydrophilic MCM of XH1 may interact preferentially with one of  $\alpha$ -helices in A $\beta$ 1-40 while its lipophilic ABMs stretch out towards hydrophobic C-terminal of A $\beta$ 1-40.

### 3.2. XH1 decreased Zn(II)-induced A $\beta$ aggregation in vitro

Zn(II) and Cu(II) binding to human A $\beta$  peptides is mainly mediated by histidine residues in A $\beta$  peptides (Bush et al., 1994; Curtain et al., 2001). It has been shown

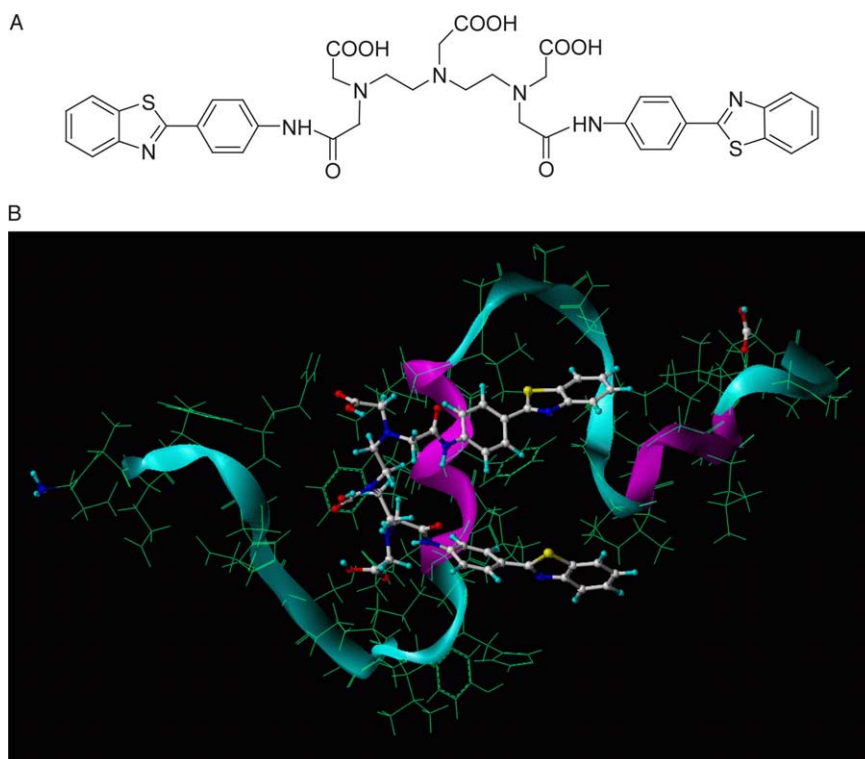


Fig. 1. Chemical structure of XH1 and its putative binding geometry with A $\beta$ 1-40. (A) The bifunctional molecule—XH1 was synthesized and characterized for its chemical identity. This symmetric molecule contains two identical ABMs and one MCM. (B) A putative binding geometry between XH1 molecule and A $\beta$ 1-40 peptide was acquired by a computational ligand/receptor docking procedure using the software package—SYBYL. The NMR structure of A $\beta$ 1-40 in 40% (v/v) trifluoroethanol/water was downloaded from the protein databank, and its backbone structure including  $\alpha$ -helices (pink), N-terminal (left), and C-terminal (right) was highlighted. The representation of XH1 chemical structure is in stick-and-ball form.

previously that metal ions such as Zn (II) and Cu(II) promoted rapid A $\beta$  aggregation in vitro, and metal chelators attenuated the effects (Bush et al., 1994; Huang et al., 1997; Atwood et al., 1998). Since XH1 has one MCM similar to DTPA, we assessed the effects of XH1 upon Zn(II)-induced A $\beta$ 1-40 using turbidometry assay. As shown in Fig. 2, XH1 reduced Zn(II)-induced A $\beta$ 1-40 aggregation in vitro by more than 50% comparable to DTPA.

### 3.3. XH1 was not neurotoxic at low micromolar concentrations and specifically reduced APP protein expression in human SH-SY5Y neuroblastoma cells

To assess the neurotoxicity of XH1, primary rat cortical neurons were incubated with XH1 at various concentrations (0, 1, 10  $\mu$ M) for 48 h, and cell viability was measured by LDH and MTT assay kits (Roche). As shown in Fig. 3, cell viability at 3 different treatment doses were all above 85%, indicating that XH1 did not induce significant neuronal cell death at low micromolar concentrations.

In human SH-SY5Y neuroblastoma cells, the effects of XH1 upon APP protein expression were measured by SDS-PAGE. The results of these experiments were reported in Fig. 4A. The figure showed that an increase in XH1 concentration led to a reduction of APP protein expression while no apparent changes in  $\beta$ -tubulin expression were

observed under the same conditions. In contrast, XH1 (up to a concentration of 10  $\mu$ M) did not affect APLP1 or APLP2 protein expression. These data demonstrated the target specificity of XH1 in suppressing APP expression. Moreover, the LDH assay data clearly implied that XH1 did not cause significant cell death at low micromolar concentrations (Fig. 4B). Therefore, observed attenuating effects of

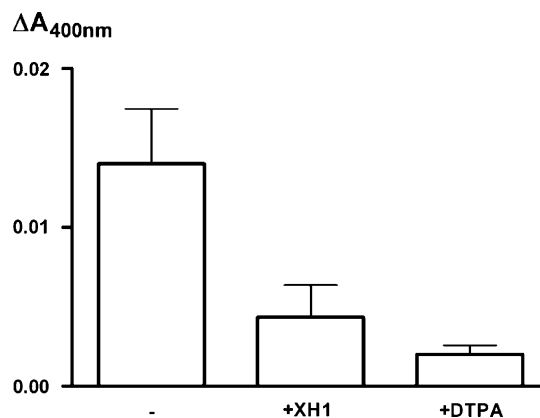


Fig. 2. XH1 reduced Zn(II)-induced A $\beta$ 1-40 aggregation in vitro. A $\beta$ 1-40 (10  $\mu$ M)/Zn(II)-histidine (10  $\mu$ M) mixture were co-incubated with or without metal chelators (XH1 or DTPA, 25  $\mu$ M) in PBS, pH 7.4, for 1 h. The data indicate the mean ( $\pm$ SD,  $n=3$ ) turbidity changes ( $\Delta A_{400\text{nm}}$ ) against the incubation buffer blank.

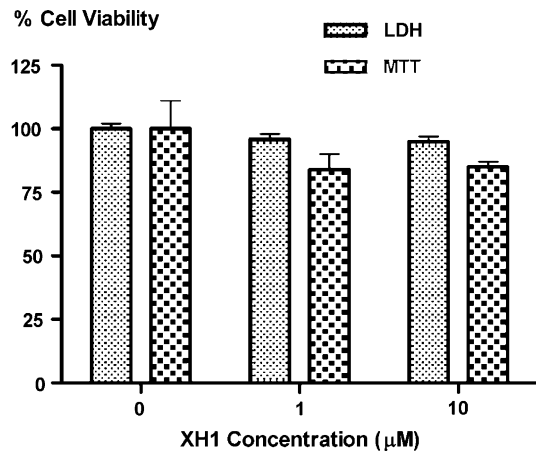


Fig. 3. XH1 was not neurotoxic at low micromolar concentrations. Primary rat neuronal cultures were exposed to XH1 (0, 1, 10 µM) for 48 h, and cell viability was measured by LDH and MTT assays. Data are means  $\pm$  SD,  $n=3$  experimental trials.

XH1 on APP protein expression were probably not due to cell death.

#### 3.4. XH1 attenuated cerebral A $\beta$ amyloid pathology in PS1/APP transgenic mice without inducing apparent toxicity and behavior disturbances

Treatment of XH1 for only 4 week already had some effects upon cerebral A $\beta$  amyloidosis in PS1/APP transgenic mice. As shown in Fig. 5A, the number of A $\beta$  amyloid plaques decreased in the XH1-untreated mouse brain. This was especially true for A $\beta$ 1-40 although both GFAP and SYP staining was not significantly different in untreated and XH1-treated mouse brain sections.

Since immunohistochemical staining for A $\beta$  peptides was only qualitative assay, we thus performed the quantitative ELISA for A $\beta$ 1-40 concentrations in the brain lysates using a commercial kit. As indicated in Fig. 5B, brain extractable A $\beta$ 1-40 concentrations showed regional

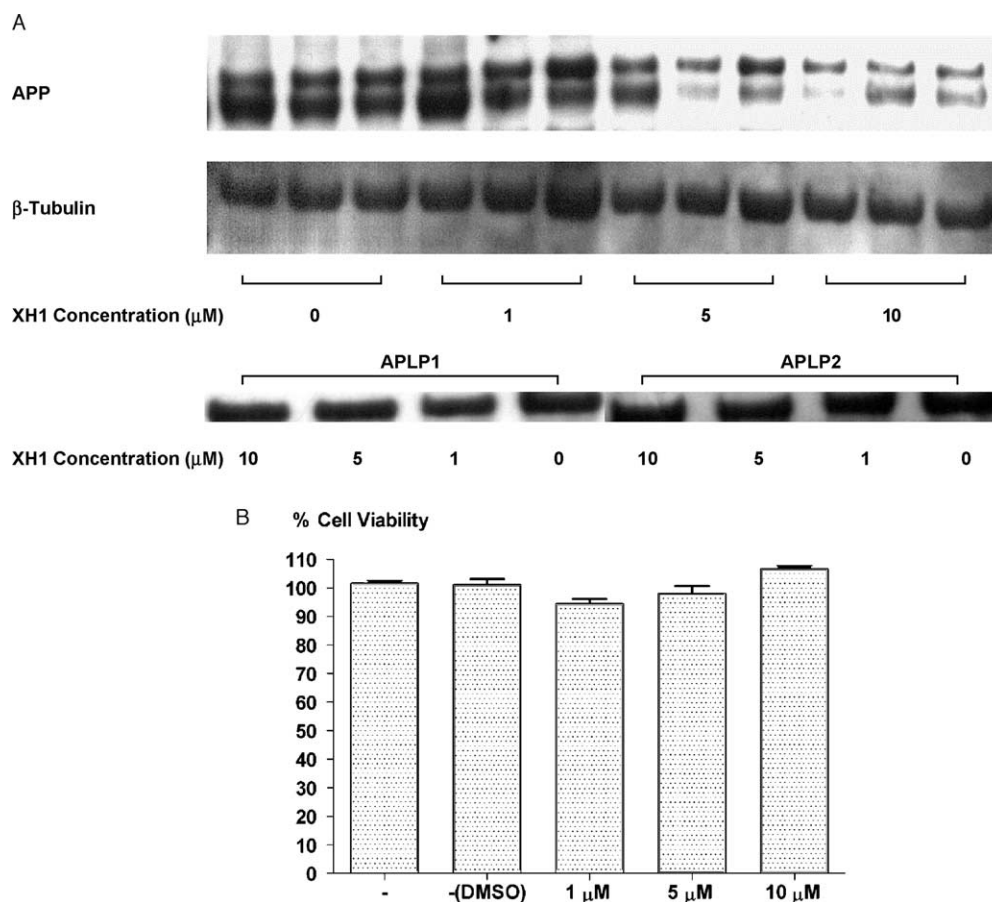


Fig. 4. XH1 specifically suppressed APP protein expression. (A) Human SH-SY5Y neuroblastoma cells were treated with XH1 at various concentrations in 100% DMSO (0, 1, 5, 10 µM) under the same cell culture conditions (95% O<sub>2</sub>, 5% CO<sub>2</sub>, 85% humidity, 37 °C) for 48 h. Cells were then washed and lysed, and endogenous expression levels of APP and other control proteins—APLP1, APLP2, and  $\beta$ -tubulin in lysates were analyzed by Western blotting. The protein bands were from three experimental trials, and cell extracts were pooled when probing APLP1 and APLP2 due to low sample quantities. (B) After 48 h treatment with XH1, SH-SY5Y cell viability was evaluated by LDH assay. Data are means  $\pm$  SD,  $n=3$  experimental trials. To minimize the potential toxic effects of DMSO on the cells, the 1000 $\times$  stock XH1 solution in 100% DMSO was prepared, and the highest DMSO concentration in the cell culture was 0.1% (v/v).

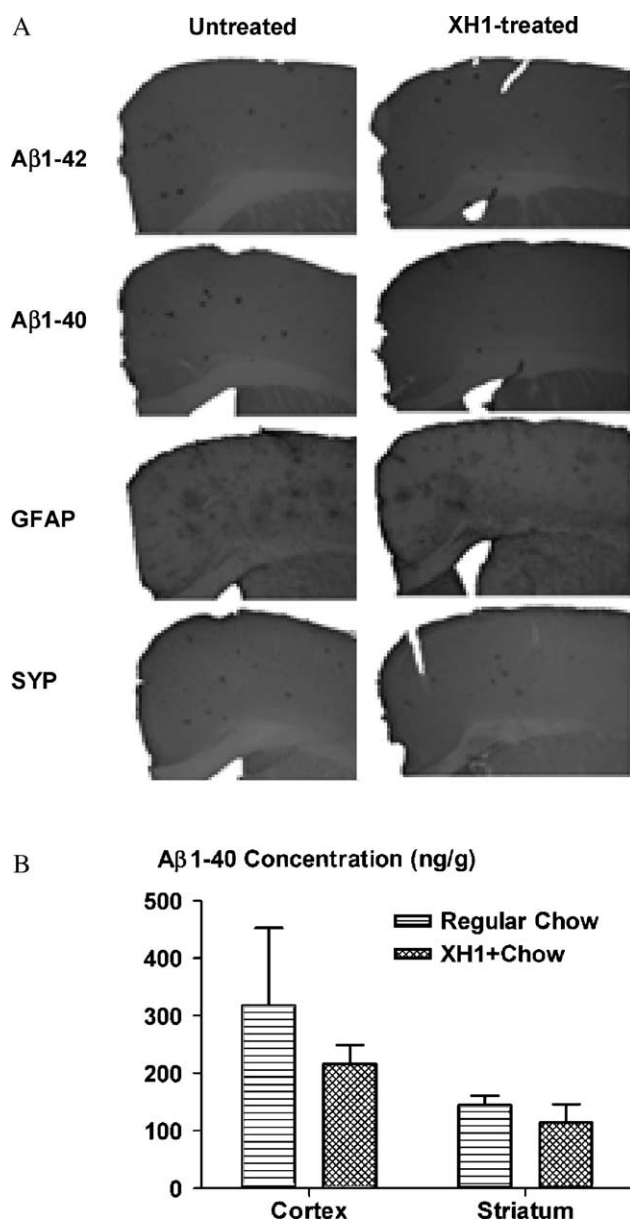


Fig. 5. XH1 attenuated cerebral A $\beta$  amyloid pathology in PS1/APP transgenic mice. (A) Representative coronal brain sections (at the anterior commissure level) from PS1/APP transgenic mice on regular and XH1-formulated diets for 4 week. They were immunostained for A $\beta$ 1-42 and A $\beta$ 1-40, illustrating the size, number and distribution of A $\beta$  amyloid plaques. The immunostaining for reactive astrogliosis marker—GFAP and negative staining for SYP, a marker for neuronal synaptic activity, were also performed. (B) Colormetric ELISA for A $\beta$ 1-40 in the brain tissue lysates from the PS1/APP transgenic mice was performed using a commercial kit (BioSource International). The concentration of A $\beta$ 1-40 was normalized to total extracted protein in the lysates.

differences in the PS1/APP transgenic mice. Average cortical A $\beta$ 1-40 concentrations were 2-fold higher than striatal ones as striatum region is not usually affected by Alzheimer's amyloid pathology in AD brain (Hyman et al., 1984). Further, corroborating with the immunostaining result of A $\beta$ 1-40, cortical extractable A $\beta$ 1-40 concentration reduced by 32% in XH1-treated PS1/APP transgenic mice.

In contrast, mean soluble A $\beta$ 1-40 concentrations in striatum tissue were not notably different in the untreated and XH1-treated transgenic mice.

#### 4. Discussion

Increasing evidence suggests that direct interplay of brain metal dysregulation, APP amyloidogenic processing, and A $\beta$  amyloidosis may be intimately involved in Alzheimer's pathology. Only recently has the importance of this interplay in AD pathogenesis begun to be recognized. Present data suggest that metals can interact directly with A $\beta$  peptide, the principle component of A $\beta$  amyloid that is one of the primary lesions in AD. The binding of metals to A $\beta$  modulates several physiochemical properties of A $\beta$  that is thought to be central to the pathogenicity of the A $\beta$  peptides. Moreover, biometals and metal chelation influence APP processing. Many mechanistic aspects of these interactions are currently unclear and further investigation is certainly required. However, an objective assessment of available experimental and clinical data clearly indicates that APP/A $\beta$ /metal interactions are among the promising therapeutic targets for current AD drug development. Nevertheless, a new generation of metal chelators need to be developed to overcome poor target specificity and unfavorable clinical safety profile of current metal-chelating agents (Huang et al., 2004).

The bifunctional XH1 molecule designed rationally from the novel 'pharmacophore' conjugation concept could be a druggable lead compound representing the new class of metal chelators. Except its molecular weight, the XH1 molecule has 2 H-bond donors (<5), 8 H-bond acceptors (<10), and 3.61 of computed log *P* value (<5), without violation of Lipinski's Rule of Five (Lipinski et al., 2001). XH1 has shown minimal cellular toxicity at low micromolar concentrations and no apparent animal toxicity during the 4 week treatment regime. More encouragingly, the predicted lipophilic property of XH1 and its attenuating effects upon cerebral A $\beta$  amyloidogenesis in PS1/APP transgenic mice may demonstrate its BBB-permeability. Further studies are needed to determine XH1 pharmacodynamics (PD) and pharmacokinetics (PK) in vivo, which includes its absorption, distribution, metabolism, excretion, and toxicity (ADMET) in vivo. Additionally, reduction of Zn(II)-induced A $\beta$  aggregation in vitro by XH1 may implicate its capability of interdicting A $\beta$ /metal interactions in vivo.

APP gene is the first AD-associated gene that has been identified and its mutations cause early onset AD (Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987, 1988). APP is also a substrate synergistically cleaved by  $\beta$ -secretase and  $\gamma$ -secretase to generate A $\beta$  peptides that accumulate as amyloid plaques in AD brain. It has been suggested a direct connection between increased levels of the APP expression and the development of AD (Selkoe, 2001). Indeed, over-expression of mutant human APP gene

in transgenic mice was found to be necessary for excessive A $\beta$  production that leads to cerebral amyloid deposition and an Alzheimer-like pathology (Hsiao et al., 1996). However, APP gene knock-out was not lethal for mice as they were viable and fertile (Zheng et al., 1995). Therefore, attenuation of APP synthesis and consequential A $\beta$  production by novel agents is an attractive therapeutic approach for AD (Huang et al., 2004).

We have previously discovered that metal chelators such as DFO, CQ, and dimercaptopropanol (DMP) at micromolar concentrations suppressed APP protein expression via their interactions with the 5'UTR of APP mRNA (Rogers et al., 2002a,b; Payton et al., 2003). Interestingly, XH1 specifically attenuated APP expression without affecting APLP1 and APLP2, the members of APP superfamily (Wasco et al., 1992, 1993). XH1 treatment also reduced extractable A $\beta$ 1-40 concentration in the cortex of PS1/APP mice. It remains to be determined whether XH1 treatment will suppress cerebral APP expression in PS1/APP mice using a human APP-specific antibody. In addition, it will be interesting to see the effects of longer treatment period of XH1 upon A $\beta$  amyloidogenesis, reactive gliosis, and synaptosis in PS1/APP transgenic mouse model.

Taken together, we have designed and characterized a novel bifunctional molecule—XH1, which may be BBB-permeable. Our pilot studies of XH1 indicate that XH1 does not have significant cellular and animal toxicity at the tested doses. It also reduces Zn(II)-induced A $\beta$ 1-40 precipitation and APP expression in vitro, and attenuates cerebral A $\beta$  amyloid pathology in PS1/APP transgenic mouse model in a short 4 week treatment period. Hence, these preliminary findings implicate the potential of XH1 as a candidate metal chelator targeting Alzheimer's amyloidogenesis and warrant further investigations.

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