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Identification of small-molecule inhibitors of the Aβ–ABAD interaction

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Abstract—The interaction of amyloid beta peptide (Aβ) and Aβ-binding alcohol dehydrogenase (ABAD) was recently implicated in the pathogenesis of Alzheimer's disease (AD). Using an ELISA-based screening assay, we identified frentizole, an FDA-approved immunosuppressive drug, as a novel inhibitor of the Aβ–ABAD interaction. Analysis of the frentizole structure–activity relationship led to identification of a novel benzothiazole urea with a 30-fold improvement in potency.

Alzheimer's disease (AD) is the most common form of senile dementia, affecting 15 million worldwide.[1] AD is characterized by progressive memory loss, decline in language skills and other signs and symptoms of cognitive impairment. Currently, no effective cure exists for this chronic debilitating illness. Current therapeutics modulate global acetylcholine-based neurotransmission and clinical benefits are modest, transient, and sporadic. A specific treatment for AD based on pathogenetic mechanisms is needed. Although the etiology of AD is not understood completely, amyloid beta-peptide (Aβ), the major component of senile plaques found in AD brains, is implicated in the pathogenesis.[2] The pathway by which Aβ causes neuronal dysfunction is the subject of intense interest and evidence supports a role for a mitochondrial alcohol dehydrogenase, alternately designated ERAD or HSD-10, found to bind Aβ in the yeast two-hybrid system and thus also known as amyloid-binding alcohol dehydrogenase (ABAD).[3] Recently, we found that Aβ can enter neuronal mitochondria to associate with ABAD, and initiate a cascade culminating in apoptosis. ABAD is upregulated in AD patients and our mice overexpressing ABAD showed memory impairment and an AD-like phenotype. These results suggest that ABAD provides a direct molecular link from Aβ to neurotoxicity. Specific interruption of the binding of Aβ to ABAD suppressed Aβ-induced neuronal dysfunction, and thus the Aβ–ABAD interaction could be a useful target for drug development.[4,5]

We sought to test this hypothesis by identifying small molecules that block the Aβ–ABAD interaction. To this end, we first developed a high-throughput screening assay for the binding of Aβ to ABAD. In a standard sandwich ELISA,[6] we found that biotin-labeled ABAD binds to fixed Aβ in a dose-dependent manner (Fig. 1). An ABAD–biotin concentration of 2.5 μM was selected for screening and, as a control, non-labeled ABAD was found to compete with a maximum inhibition of 77.4% at 25 μM (Fig. 2, ABAD aggregation interferes with the assay at higher concentration).

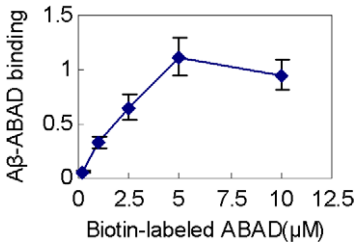

Figure 1. The ELISA-based assay of the binding of Aβ–ABAD. Dose-dependent binding of biotinated ABAD to Aβ. Ninety-six-well plates loaded with 5 μg of Aβ were incubated with biotin-labeled ABAD at varying concentrations with detection via ExtrAvidin-peroxidase absorbance at 490 nm. The binding was corrected for background in the absence of Aβ. The assay was run in triplicate and plated as mean ISD.

Keywords: Aβ–ABAD interaction; Alzheimer's disease; ELISA; Benzothiazole urea.
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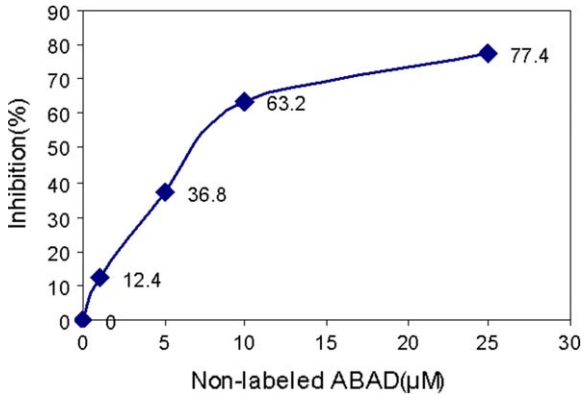


Figure 2. Inhibition of the binding with non-labeled ABAD. The binding of biotinated ABAD (2.5 μM) to fixed Aβ was determined in the presence of varying concentrations of free ABAD. The inhibition (%) = [binding without free ABAD—binding with free ABAD]/binding without free ABAD] × 100%.

With a convenient assay in hand, we initially screened a focused in-house chemical library of 50 commercially available compounds that (1) interact with Aβ and/or ABAD such as amyloid-binding dyes, AD imaging agents, cyclodextrins, NAD^+ (ABAD enzyme cofactor), and nucleotides, or (2) are considered neuroprotective (for the possibility that this neuroprotection represented AB or ABAD binding activity). The initial screen identified three inhibitors: Congo red, thioflavine T, and resveratrol with inhibition at 100 μM of 100%, 21.0%, and 27.9%, respectively. The Aβ binder Congo red was eliminated due to high toxicity and poor cell permeability, and analogs of the neuroprotective agent resveratrol had no activity in our assay. Thus, our attention turned to thioflavine T, a nontoxic amyloid-binding dye. A secondary screen of thioflavine T analogs identified frentizole, a benzothiazole urea, as the most promising hit (Fig. 3).

Frentizole, a nontoxic antiviral and immunosuppressive agent used clinically in rheumatoid arthritis and systemic lupus erythematosus, displayed a slightly improved activity (IC_{50} = 200 μM) compared to thioflavine T (IC_{50} = 230 μM). More importantly, frentizole allowed facile construction of a library for SAR analysis and we synthesized a series of analogs with variations at the aromatic rings and their linking group. As shown in Scheme 1, benzothiazole amines (1), either commercially available or prepared from the corresponding phenylamines, were treated with acid chlorides in the presence of excess triethylamine in THF at room temperature to yield the corresponding amides (2). In parallel, the 2-amino-benzothiazoles were reacted with a series of imidazole carbamates or thiocarbamates, and then with aromatic amines in DMF at 100 °C to yield the corresponding ureas (**3**, **5**, and **6**) and thioureas (**4**) (Scheme 1).

A total of 45 compounds were synthesized,[7] and their capacity to inhibit Aβ–ABAD binding was measured by ELISA as described above, with IC_{50}s calculated by SigmaPlot from a five-point dose–effect curve run in duplicate (Table 1). The preliminary structure–activity relationship (SAR) study indicated that the urea moiety was required for inhibitory activity. Amide compounds (**2a**–**2k**) showed less than 50% inhibition at high concentration (1 mM), while the ureas completely blocked Aβ–ABAD binding at 1 mM (data not shown). We speculated that hydrogen bond donation at the urea participated Aβ or ABAD binding and consistent with this hypothesis, the corresponding thioureas retained potency. Replacement of the phenylurea ring with a heterocyclic (Series 3 compounds) or polycyclic structure (Series 6 compounds) resulted generally in lower inhibition. Substitutions on the benzothiazole and phenylurea rings dramatically affected potency. Small electron-withdrawing groups were preferred at the benzothiazole ring with Cl and F particularly favored. Also, compounds with a hydroxyl group at the para position of the phenylurea were noticeably more potent. Combining these features resulted in our two most potent inhibitors, **5h** and **5l**, with IC_{50}s of <10 μM each.

In summary, we have successfully identified a class of benzothiazole ureas as micromolar inhibitors of the Aβ–ABAD interaction. The compounds **5h** and **5l** are presently the most potent inhibitors discovered in this study. As low-molecular-weight compounds, with octanol–water partition coefficients (log *P*) of 1.34 and 1.15,[8] respectively, they are likely to cross the blood–brain barrier adequately. In the future, improved potency will be sought through an expanded library of benzothiazole ureas. If adequate CNS penetration can be demonstrated, as a proof-of-concept, the inhibitors will be tested in our AD animal models. Further studies to characterize CNS penetration of **5h** and **5l** are in progress.

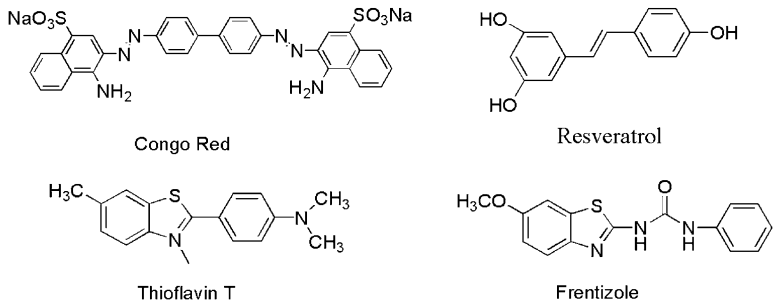
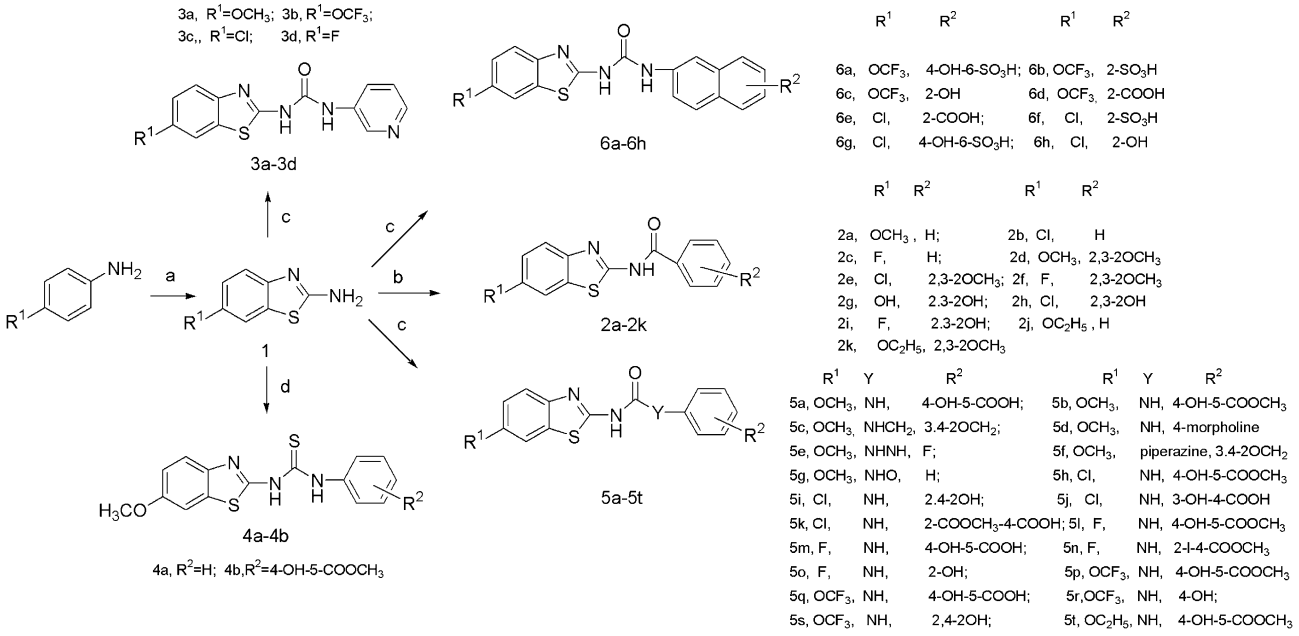


Figure 3. The chemical structures of the hits.



Scheme 1. Synthesis of frentizole analogs. Reagents and conditions: (a) i—NH_4SCN, HCO_2H/HAc (1:4), 0 °C, 0.5 h; ii—Br_2, 0 °C, 5 h; (b) substituted benzoyl chloride, THF, rt; (c) i—1,1′-carbonyldiimidazole, CH_3CN, rt; ii—amines, DMF, 100 °C; (d) i—1,1′-thiocarbonyldiimidazole, CH_3CN, rt; ii—amines, DMF, 100 °C.

Table 1. Inhibition of the Aβ–ABAD interaction determined by ELISA[a]

Compound	IC_{50} (μM)
3a	256.7
3b	243.8
3c	247.3
3d	282.5
4a	190.5
4b	15.6
5a	29.5
5b	26.3
5c	26.6
5d	250.0
5e	154.1
5f	162.9
5g	316.2
5h	6.46
5i	23.0
5j	267.3
5k	22.7
5l	6.56
5m	260.6
5n	286.4
5o	207.0
5p	244.3
5q	184.9
5r	47.6
5s	53.8
5t	20.6
6a	227.0
6b	280.5
6c	212.3
6d	18.2
6e	27.9
6f	29.0
6g	267.3
6h	36.3

[a] IC_{50} is defined as concentration of compounds to obtain 50% of maximum inhibition (100%).

Acknowledgments

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References and notes

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6. ELISA protocol: 96-well plates were coated with commercially available Aβ1–42 (5 μg per well) and then blocked with 2% of BSA (150 μL per well). After washing, different amount of biotin-labeled recombinant ABAD was added followed by incubation at 37 °C for 2.5–3 h. The amount of ABAD bound to the plates was detected using ExtrAvidin-Peroxidase (Sigma) according to the protocol provided by the company. For measuring the inhibitory activity, the testing agents were added to the plates before addition of biotin-labeled ABAD.
7. Analytical data for representative compounds: **2d**, ^{1}H NMR ($CDCl_3$) ppm 3.91 (s, 3H, OCH_3); 3.93 (s, 3H, OCH_3); 4.10 (s, 3H, OCH_3); 7.0–7.31 (m, 2H, Ph-H); 7.80 (dd, 1H, Ph-H); ESI-MS (M^++1): 360.4. Compound **3d**, ^{1}H NMR (DMSO-*d*) ppm: 7.15–7.30 (d, 1H, Ph-H); 7.31–7.40 (m, 1H, Py-H); 7.58–7.69 (m, 1H, Py-H); 7.75–7.85 (m, 1H, Ph-H); 7.95–8.00 (d, 1H, Ph-H); 8.20–8.30 (d, 1H, Py-H); 8.53 (s, 1H, Py-H); ESI-MS (M^++1): 289.0. Compound **5h**, ^{1}H NMR (DMSO-*d*) ppm: 3.85 (s, 3H, $-COOCH_3$); 6.90–6.98 (d, 1H, Ph-H); 7.35–7.42 (d, 1H, Ph-H); 7.51–7.62 (m, 2H, Ph-H); 8.03 (br, 2H, Ph-H); 9.10 (s, 1H, OH); ESI-MS (M^++1): 377.9. Compound **6a**, ^{1}H NMR (DMSO-*d*) ppm: 7.10 (s, 1H, Naph-H);

7.35–7.40 (d, 1H, Naph-H); 7.48 (s, 1H, Naph-H); 7.55–7.62 (d, 1H, Ph-H); 7.70–7.79 (d, 1H, Naph-H); 7.95 (s, 1H, Naph-H); 8.01–8.05 (d, 1H, Ph-H); 8.06 (s, 1H, Ph-H); 9.35 (s, 1H, OH); ESI-MS ($M^- - 1$): 498.0.

8. The octanol–water partition coefficient log *P* of **5h** and **5l** was experimentally determined as described in the literature: Rothwell, J. A.; Day, A. J.; Morgan, M. R. *J. Agric. Food Chem.* **2005**, *53*, 4355.