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Design, synthesis and *in vitro* evaluation of benzothiazole-based ureas as potential ABAD/17β-HSD10 modulators for Alzheimer's disease treatment

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Abstract

Amyloid-beta peptide (Aβ) has been recognized to interact with numerous proteins, which may lead to pathological changes in cell metabolism of Alzheimer's disease (AD) patients. One such known metabolic enzyme is mitochondrial amyloid-binding alcohol dehydrogenase (ABAD), also known as 17β-hydroxysteroid dehydrogenase type 10 (17β-HSD10). Altered enzyme function caused by the Aβ-ABAD interaction, was previously shown to cause mitochondrial distress and a consequent cytotoxic effect, therefore providing a feasible target in AD drug development. Based on previous frentizole derivatives studies, we report two novel series of benzothiazolyl ureas along with novel insights into the structure and activity relationships for inhibition of ABAD. Two compounds (**37**, **39**) were identified as potent ABAD inhibitors, where compound **39** exhibited comparable cytotoxicity with the frentizole standard; however, one-fold higher cytotoxicity than the parent riluzole standard. The calculated and experimental physical chemical properties of the most potent compounds showed promising features for blood-brain barrier penetration.

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Keywords

Alzheimer's disease (AD), amyloid-beta peptide (Aβ), mitochondria, amyloid binding alcohol dehydrogenase (ABAD), 17β-hydroxysteroid dehydrogenase type 10 (17β-HSD10), benzothiazole, riluzole

Alzheimer's disease (AD) is the most common form of dementia in the elderly, characterized by the slow deterioration of cognitive functions.[1] The accumulation of amyloid-beta peptide (Aβ) and its formation into plaques, along with the formation of neurofibrillary tangles, highlights the pathological changes in affected brain regions with progressed AD. These hallmarks have become commonly monitored markers, however the initiating events still remain unclear.[2] Even though a precise mechanism of Aβ-induced toxicity has not been fully understood, several studies have reported synaptic and mitochondrial Aβ accumulation and dysfunction in early stages of AD development.[2–4] Furthermore, it has been described that Aβ interacts with various mitochondrial proteins, resulting in enhanced oxidative stress, energy misbalance and overall cell toxicity.[2,5–8]

Amyloid-binding alcohol dehydrogenase (ABAD), also known as 17β-hydroxysteroid dehydrogenase type 10 (17β-HSD10), is one of the proteins which was identified to interact directly with Aβ at nanomolar concentrations.[9,10] Moreover, it has been reported that the interaction between Aβ and ABAD promotes oxidative stress and mitochondrial dysfunction, consequently resulting in cell death.[5] Cell based assays and transgenic mice experiments demonstrated that the overexpression of both Aβ and ABAD show enhanced cell cytotoxicity, reduced levels of ATP and COX activity along with impaired energy metabolism in mice. Conversely, the overexpression of Aβ with inactive ABAD displayed less cytotoxicity and transgenic (ABAD) mice when compared with non-transgenic mice do not display these changes.[7,10] Lim *et al.* reported an ABAD inhibition study where the compound (AG18051) appeared to reduce the levels of Aβ-induced oxidative stress and mitochondrial respiration impairment, as well as alleviate the Aβ-induced down-regulation of ABAD activity.[11] Whereas elevated levels of ABAD have been associated with AD pathology.[10,12] Reduced levels of ABAD has been reported in brain of Parkinson's disease patients.[13] These findings suggest that both ABAD-Aβ interaction and ABAD itself may represent a viable objective for deeper understanding of AD pathogenesis in context of Aβ-induced toxicity. Consequently, it may also aid in the development of novel AD therapeutics.

There has been a limited number of reported compounds acting as ABAD or ABAD-Aβ interaction inhibitors.[14,15] In 2006, Xie *et al.* described the marketed drug frentizole acting as a poor inhibitor of ABAD-Aβ interaction (IC_{50} ~200 μM) along with a novel series of synthesised frentizole analogues displaying a 30-fold increase in improved potency (IC_{50} ~6.5 μM).[16] A more recent study reported two phosphonate analogues of these previously discussed compounds, which also exhibited moderate to

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weak ABAD inhibition (IC_{50} 53 μM and 342 μM).[17,18] Thus, our aim of the study was focused on generating more potent inhibitors based around the benzothiazole scaffold. To this end, a first series was designed around a 6-halogen-benzothiazole urea scaffold with various phenyl ring substitution patterns including those previously reported (Scheme 1).[16–18] Furthermore, many benzothiazole analogues have been shown to possess various biological activities in the central nervous system.[19] Riluzole, a well-known drug with neuroprotective properties, has a similar benzothiazolyl core, however its neuroprotective mechanism of action is not still completely understood.[20] Riluzole is now an FDA approved drug to treat amyotrophic lateral sclerosis, and several other studies report riluzole exhibiting neuroprotective properties in other neurological disorders (e.g. Parkinson's disease, Huntington's disease or cerebral ischemia).[21–23] Riluzole possesses a wide range of mechanisms of action including anti-glutamate activity, Na^+ and Ca^{2+} channel blockage, GABAergic modulation[20] throughout the modulation of the excitatory cascade.[24] As Aβ is known to disturb cell ionic homeostasis on various levels including calcium balance,[25] it can be hypothesized that a riluzole moiety may partially mitigate such Aβ-induced ionic homeostasis misbalance. Thus, a second series of compounds was designed with a riluzole core moiety and further combined with a urea linker and substituted phenyl moiety to more closely address the effect of phenyl ring substitution variations (Scheme 1). The urea linker was selected based on compounds that were found to be the most potent in perturbing the ABAD-Aβ interaction.[16] Additionally, the phenyl substitutions were based upon the first series of compounds, where oxygen-based substitutions with additional halogen substitutions were selected. The second series includes both overlapping substitutions of the first compound series (to confirm possible lead structures and validate structure-activity relationship). More importantly additional variations of the phenyl ring were made to more exhaustively explore possible phenyl ring variations and structure-activity relationship necessary for ABAD inhibition.

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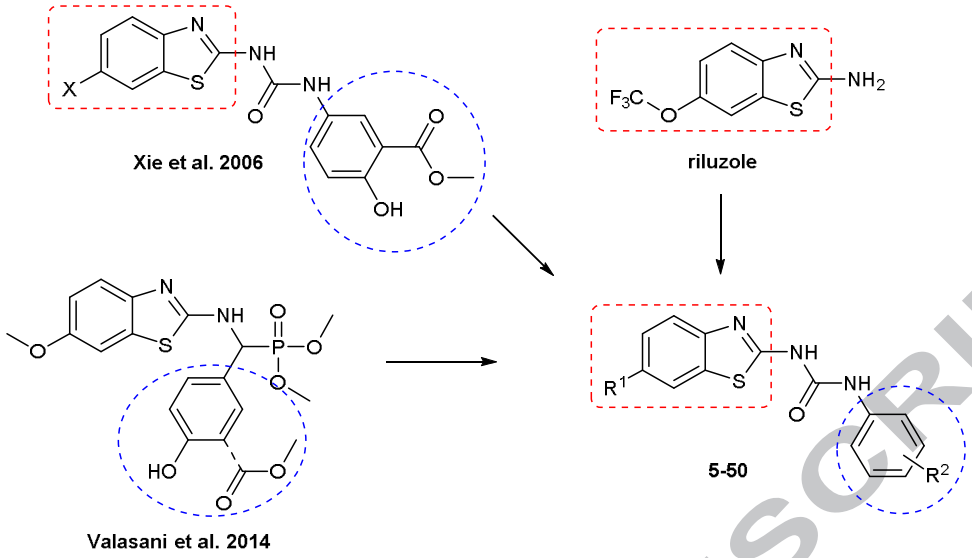
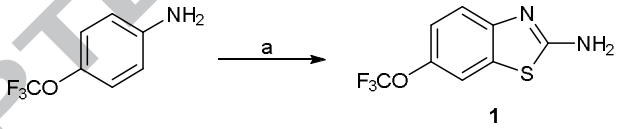


Scheme **1**. Design of benzothiazole-based urea ABAD inhibitors.

While commercially available 6-flouro and 6-chloro substituted benzo[*d*]thiazole-2-amines were used in the first series of compounds, 6-(trifluoromethoxy)benzo[*d*]thiazol-2-amine (riluzole) was prepared from its corresponding *para*-substituted aniline derivative (Scheme 2). Therefore, 4-(trifluoromethoxy)aniline was treated with potassium thiocyanate and bromine to afford 6-(trifluoromethoxy)benzo[*d*]thiazol-2-amine (**1**) in excellent yield (94%).[26]



Scheme 2. Synthesis of 4-(trifluoromethoxy)aniline **1**. Reagents and conditions: (a) Br_2, KSCN, CH_3COOH.

Subsequently, two series of benzothiazolyl ureas **5-50** were prepared in a two-step synthesis (Scheme 3). Selected 6-subtituted benzo[*d*]thiazole-2-amines were activated with *N,N'*-carbonyldiimidazole to obtain intermediates **2-4** in excellent yields (90-95%), followed by subsequent treatment with a substituted aromatic amine[16] to produce final benzothiazole ureas **5-50** (Table 1) in moderate to excellent yields (36-99%).

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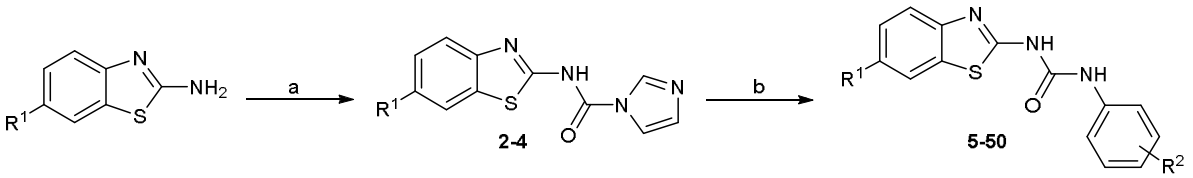

Scheme 3. Synthesis of benzothiazolyl ureas **5-50**. Reagents and conditions: (a) CDI, DCM, reflux; (b) Ar-NH_2, MeCN, reflux.

A series of compounds (**5-50**) was screened at 25 µM to determine their ABAD inhibitory ability (Table 1) and to show their structure-activity relationship. Within the first series neither frentizole nor compounds **5, 6, 11** and 1**5** (as with previously reported substitutions[16–18]) showed any noteworthy ABAD inhibitory ability. However, compounds **13** and **14** showed a substantial decrease (˜60%) in ABAD activity. Furthermore, compound **12** showed a notable decrease (˜20%) in ABAD activity. Therefore, selected substitutions were included in the second riluzole-based series with additional phenyl ring substitution patterns. The *para*-hydroxy along *meta*-chlorine substitution patterns proved to display the most pronounced inhibitory activity, where compound **37** showed a significant decrease (˜80%) in ABAD activity. Moreover, the second chlorine in close proximity of the phenolic group (**39**) showed comparable inhibitory activity with compound **37**. Therefore, the presence of the free phenolic group attached to the distal phenyl ring, is shown to be essential for its ability to inhibit ABAD, and the addition of an electron-withdrawing group (such as chlorine) in its adjacent proximity greatly increases such an inhibitory ability. Other compounds with a single phenolic group (**12**, **33-35**), displacement of 3-chloro-4-hydroxy pattern (**36**, **38**) or isosteric change of hydroxyl to thiol group (**50**) showed a less pronounced drop in ABAD activity (10-30 %). However, such a low decrease in enzymatic activity could be accounted by an experimental error. Thus these substitution patterns did not provide sufficient SAR information, but they rather indicate the presence of weak ABAD inhibition. Interestingly, 3,4-dihydroxy substitution displayed a similarly low decrease in enzyme activity (˜20%). On the other hand, compound **41** revealed an approximately 20% increase in ABAD activity, which will be further investigated. Both compounds **37** and **39** demonstrated a two-fold decrease in ABAD activity when compared to compounds **13** and **14**, thus underlining that the introduction of a 6-trifluromethoxy moiety instead of a 6-halogen moiety, led to an increased inhibitory ability towards ABAD. IC_{50} values were attempted to be determined for compounds **37** and **39**, however, this data was unable to be obtained due to the limited solubility of these compounds within the enzyme activity assay, and due to the possibility that a biophysical effect was observed rather than a true ligand-protein interaction.

Table 1. Prepared benzothiazole ureas (**5-50**) and their *in vitro* evaluation.

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Comp.	R^1	R^2	Relative % activity remaining @25 µM[1]
Control	-	-	100 ± 1.2
Frentizole	OCH_3	-	97.9 ± 3.6
5	F	3-COOMe, 4-OH	97.2 ± 3.8
6	Cl	3-COOMe, 4-OH	95.6 ± 4.5
7	F	4-OH	94.4 ± 1.1
8	Cl	4-OH	91.9 ± 1.9
9	F	3-OH	94.8 ± 1.0
10	Cl	3-OH	93.6 ± 1.9
11	F	2-OH	91.5 ± 1.5
12	Cl	2-OH	77.1 ± 1.0
13	F	3-Cl, 4-OH	39.8 ± 0.5
14	Cl	3-Cl, 4-OH	38.6 ± 0.7
15	F	3-COOH, 4-OH	101.3 ± 1.6
16	Cl	3-COOH, 4-OH	100.5 ± 1.1
17	F	4-OMe	110.9 ± 1.8
18	Cl	4-OMe	104.6 ± 1.2
19	F	3,4-OMe	103.6 ± 1.9
20	Cl	3,4-OMe	104.4 ± 1.5
21	F	3-COOH, 4-OMe	105.5 ± 1.5
22	Cl	3-COOH, 4-OMe	107.2 ± 2.5
23	F	4-OPh	108.8 ± 2.2
24	Cl	4-OPh	107.4 ± 1.9
25	F	4-COOH	107.8 ± 1.7
26	Cl	4-COOH	105.5 ± 1.7
27	F	4-COOEt	110.3 ± 1.7
28	Cl	4-COOEt	108.6 ± 2.2
29	F	4-COOMe	114.7 ± 5.3
30	Cl	4-COOMe	99.3 ± 3.2
31	F	4-NHCOMe	94.7 ± 3.3
32	Cl	4-NHCOMe	97.9 ± 3.4
33	OCF_3	2-OH	92.0 ± 0.1
34	OCF_3	3-OH	78.8 ± 0.1
35	OCF_3	4-OH	69.6 ± 0.1
36	OCF_3	2-Cl, 4-OH	92.0 ± 1.6
37	OCF_3	3-Cl, 4-OH	17.3 ± 0.8
38	OCF_3	2-OH, 4-Cl	65.5 ± 3.0
39	OCF_3	3,5-Cl, 4-OH	20.3 ± 1.1
40	OCF_3	3-Cl, 4-COOH	104.8 ± 2.6
41	OCF_3	2-OH, 4-COOH	119.8 ± 2.1
42	OCF_3	3-OH, 4-COOH	94.8 ± 5.6

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43	OCF_3	3-COOH, 4-OH	96.6 ± 5.5
44	OCF_3	4-OMe	100.7 ± 2.4
45	OCF_3	3-Cl, 4-OMe	91.0 ± 2.6
46	OCF_3	3-OH, 4-OMe	98.3 ± 6.1
47	OCF_3	3,4-OMe	78.9 ± 5.9
48	OCF_3	3-COOH, 4-OMe	86.8 ± 3.0
49	OCF_3	4-COOMe	98.2 ± 1.8
50	OCF_3	2-SH	80.6 ± 5.0

[1] Relative remaining activity is displayed in percentage of 3 independent measurements ± SEM.

A key aspect in the design of any potential ABAD inhibitors is that they should be CNS penetrable. Thus, essential physical chemical properties (HBA, HBD, TPSA, ClogP, ClogD) for efficient blood-brain barrier (BBB) penetration were calculated for the two most promising inhibitors **37** and **39** using ACDLabs PhysChem Suite 2014 (Table 2). The calculated values of the selected physical chemical properties either oscillate close to the upper threshold or were found to be slightly higher than optimal values proposing CNS bioavailability. Additionally, logP and logD values (further referred as ElogP and ElogD) were experimentally determined using adapted OECD guideline.[27] Both calculated values of ClogP and ClogD were found to be in very good agreement with experimental data. The presented physical chemical properties of **37** and **39** showed reasonable conformity with the requirements for centrally acting compounds[28] (based on earlier defined Lipinski rule of five for orally administrated drugs).[29] Experimental prediction of BBB penetration is further shown in Table 2. Data obtained for **37** and **39** are correlated to standard drugs (e.g. donepezil) , where CNS availability is known and also reported using the PAMPA assay (Supporting information, Table S1).[30] The acquired data from the PAMPA assay predicted a possible high BBB penetration for compound **39**, whereas experimental data for compound **37** estimated a rather uncertain BBB penetration.

Table 2. Physicochemical properties of **37** and **39**.

Comp.	Mw	HBA/HBD	TPSA (Å^2)	ClogP ± SD[1]	ELogP ± SD[2]	$ClogD_{7.4}$[1]	$ELogD_{7.4}$ ± SD[2]	P_e ± SEM[3]
Optimal LR5 [28,29]	≤ 450	≤ 7 / ≤ 3	≤ 90	≤ 5	≤ 5	0-3	0-3	-
donepezil	379.49	4/0	38.8	4.23 ± 0.40	---	2.79	---	7.3 ± 0.9
37	403.76	6/3	111.7	4.24 ± 0.41	4.07 ± 0.11‰	3.35	4.04 ± 2.16‰	2.2 ± 0.4
39	438.20	6/3	111.7	5.18 ± 0.43	4.65 ± 0.43‰	3.79	4.40 ± 2.17‰	7.0 ± 1.4

[1] The values were calculated by ACDLabs PhysChem Suite 2014.
[2] The values refer to experimentally acquired data.
[3] Prediction of blood-brain barrier penetration of drugs expressed as Pe ± SEM (n = 2-4). High BBB permeation predicted for Pe > 4; BBB permeation uncertain for Pe between 2.0 and 4.0; low BBB permeation predicted for < 2.0

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The two most potent compounds (**37**, **39**) were assayed for acute cytotoxicity evaluation (Table 3). The compounds' acute cytotoxicity was determined via a combined MTT and LDH assay using CHO-K1 cell lines. A combined cytotoxicity assay was chosen due to the MTT test being partially dependent on the mitochondrial oxidoreductases,[31] whose activity may be influenced by compounds targeted to mitochondria. Both MTT and LDH assays for compound **37** showed a greater cytotoxicity when compared to frentizole, whereas compound **39** displayed comparable cytotoxicity level with frentizole. All evaluated benzothiazolyl ureas (frentizole and compounds **37**, **39**) showed one order of magnitude higher acute cytotoxicity compared to riluzole. In general, the MTT assay data showed a trend of a slightly lower IC_{50} value compared to the LDH data. However, this fact may be caused by the compounds' involvement in the mitochondrial electron transport chain and requires to be further elucidated.

Table 3. Acute cytotoxicity evaluation of compound **37** and **39**.

Comp.	IC_{50} ± SEM[1](μM)	
	MTT CHO-K1	LDH CHO-K1
frentizole	31 ± 3	46 ± 6
riluzole	310 ± 33	480 ± 45
37	7.7 ± 0.7	9.0 ± 1.7
39	31 ± 5	41 ± 12

[1] The IC_{50} value refers to 3 independent measurements ± SEM.

In summary, two novel series of benzothiazolyl ureas were designed and synthesised. All compounds were evaluated for ABAD inhibitory ability *in vitro*, where two riluzole-based compounds (**37**, **39**) showed the most promising ABAD inhibitory activity. In terms of the structure-activity relationship for the benzothiazole-urea-phenyl scaffold, the combination of a 4-phenolic moiety with a chlorine in its close proximity was confirmed to be essential for compounds ABAD inhibitory ability (**13**, **14**, **37**, **39**). Possible good BBB permeation was predicted for compound **39** by experimental determination, and the cytotoxicity of compound **39** was also found to be similar to the frentizole standard, but was one order of magnitude higher to its parent compound riluzole. Overall the most promising compounds (**37** and **39**) exhibited solubility issues within the activity assay and were predicted with at least a borderline BBB penetration.

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Graphical abstract

Design, synthesis and in vitro evaluation of benzothiazole-based ureas as potential ABAD/17β-HSD10 modulators for Alzheimer's disease treatment

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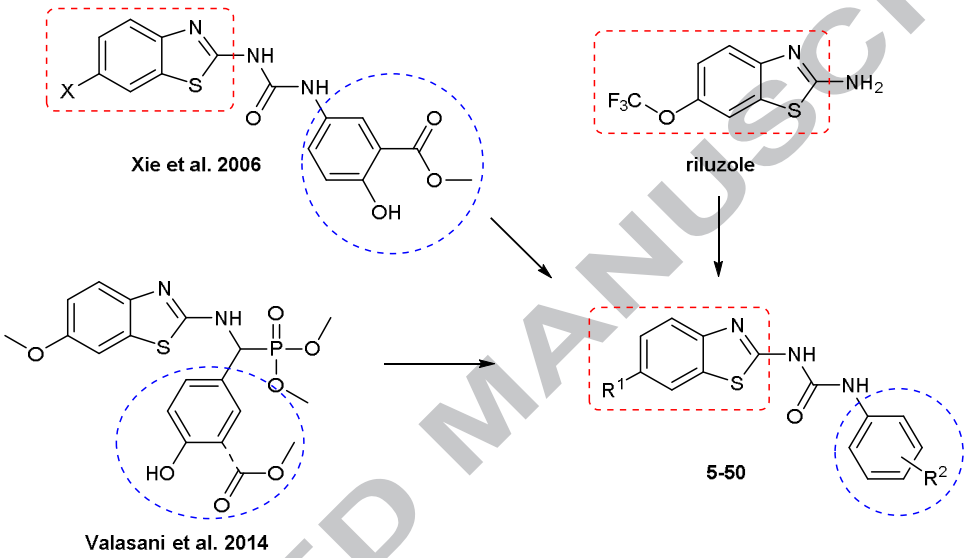

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