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The design and synthesis of human branched-chain amino acid aminotransferase inhibitors for treatment of neurodegenerative diseases

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Dedicated to the memory of David W. Robertson, mentor, colleague and friend.

Abstract—The inhibition of the cytosolic isoenzyme BCAT that is expressed specifically in neuronal tissue is likely to be useful for the treatment of neurodegenerative and other neurological disorders where glutamatergic mechanisms are implicated. Compound **2** exhibited an IC_{50} of 0.8 μM in the hBCATc assays; it is an active and selective inhibitor. Inhibitor **2** also blocked calcium influx into neuronal cells following inhibition of glutamate uptake, and demonstrated neuroprotective efficacy in vivo. SAR, pharmacology, and the crystal structure of hBCATc with inhibitor **2** are described.

The cytosolic form of branched-chain aminotransferase (BCATc), expressed in neuronal tissue, catalyzes the transfer of an amino group from branched chain amino acids (Leu, Ile, and Val) to α-ketoglutarate forming glutamate. This appears to be a major neuronal biosynthetic pathway for the de novo synthesis of glutamate.[1] It has been reported that leucine contributes ~25% of the nitrogen used for glutamate synthesis in the CNS.[2] BCATc is highly expressed in the CNS and its expression is regulated.[1] Therefore, inhibition of BCATc offers an opportunity to slow glutamate synthesis and reduce the amount of glutamate released during excitation in neuronal tissues.[2,3] The inhibition of BCATc is likely to be useful for the treatment of a wide variety of neurodegenerative and behavioral disorders where glutamatergic mechanisms have been implicated.[2,3] The mitochondrial isoenzyme, BCATm, is more ubiquitously expressed and is found in muscle, heart, and other peripheral tissues. BCATm is also expressed in brain but only probably in glia.[3] In brain its activity is only about 1/4 that of BCATc. We have developed a series of sulfonyl hydrazides, which were derived from a high-throughput screen (HTS) hit (**1**). In this report, the synthesis and SAR of these analogs and their ability to block BCATc are described (see Fig. 1).

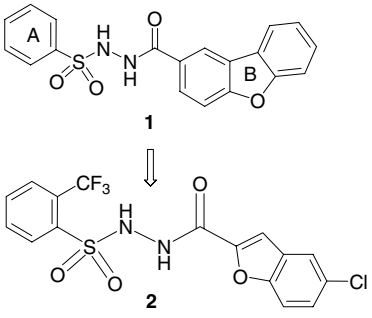

Figure 1. Inhibitors **1** and **2**.

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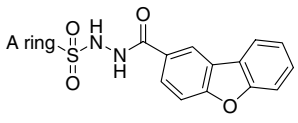
Compound **1** exhibited a moderate ability to inhibit hBCATc (IC_{50} = 2.35 μM)[4] and moderate selectivity for hBCATc versus hBCATm. We, therefore, designed a series of analogs, aimed at improving selectivity and solubility for in vivo proof-of-concept studies. An SAR study of ring A of inhibitor **1** was conducted verifying the effect of simple substitution. Among the Me-substituted derivatives, ortho- (**3**) and meta- (**4**) substitution is active while para-substitution (**5**) is not. The Cl-substituted analogs are less potent than their methyl counterparts, with the 2-Cl derivative (**6**) being more potent than the 3-Cl (**7**) or 4-Cl (**8**) compound (see Tables 1 and 2).

Further modification of compound **1** focused on replacing the dibenzofuran ring with smaller ring systems to improve potency and solubility for in vivo studies. We truncated the 4-dibenzofuran moiety by eliminating one of the benzene rings, which yielded the 2-benzofuran (**9**) with decreased hBCATc activity (IC_{50} = 18 μM). To improve the solubility of **1**, we modified the 2-benzofuran with the nitrogen-containing derivatives (2-indole (**10**), 3-quinoline (**11**), and 7-quinoline (**12**)). These nitrogen-containing molecules showed lower hBCATc activity. Modeling a series of analogs in the active site with SYBYL[10a] suggested that smaller substituents at the 2-benzofuran 5-position could fill a depression in the active-site surface. Therefore, the 5-bromo-2-benzofuranyl (**13**), 5-chloro-2-benzofuranyl (**14**), and 5-methoxy-2-benzofuranyl (**15**) analogs were compared. The 5-bromo and 5-chloro derivatives were the preferred inhibitors with similar potency to the 4-dibenzofuran analog (**1**). In the 2-indole series, the 5-bromo-2-indole (**16**) was more potent than the 5-methoxy-2-indole (**17**) (see Table 3).

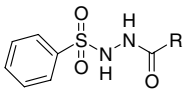
With the above findings, we considered that both the solubility and activity of our BCATc inhibitors were critical to the in vivo proof-of-concept study. We selected 5-chloro-2-benzofuran as the preferred group for the B-ring. We incorporated 2-Me-, 3-Me-, 2-Cl-, and 2-CF_3-phenyls as the A ring and constructed inhibitors **18**–**20** and **2**.[9] Among the four of them, the CF_3 analog (**2**) with an IC_{50} 0.81 μM, was the most potent inhibitor in this series.

Table 1. SAR of the A ring of compound **1**



Compound	A ring	hBCATc IC_{50}, μM (n = 2–4)
1	Phenyl	2.35 ± 0.01
3	2-Me-Phenyl	3.2 ± 0.02
4	3-Me-Phenyl	2.8 ± 0.15
5	4-Me-Phenyl	51.0 ± 10.8
6	2-Cl-Phenyl	12.9 ± 0.9
7	3-Cl-Phenyl	>35
8	4-Cl-Phenyl	37.2 ± 3.9

Table 2. SAR of the B ring of compound **1**



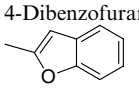
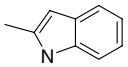
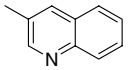
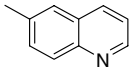
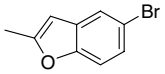
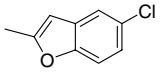
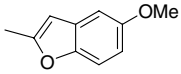
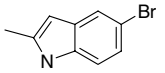
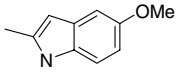
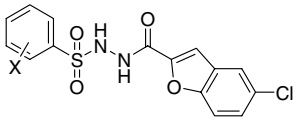
Compound	A ring	HBCATc IC_{50}, μM (n = 2–4)
1	4-Dibenzofuran	2.35 ± 0.01
9		18.3 ± 5.0
10		36 ± 2.2
11		33.3 ± 8.3
12		13.3 ± 2.5
13		2.5 ± 0.07
14		4.2 ± 0.45
15		12.8 ± 2.8
16		15 ± 2.7
17		56.8 ± 0.7

Table 3. BACTc inhibitors



Compound	X	hBCATc IC_{50}, μM (n = 2–4)	RLMT1/2 (min)
18	2-Me	1.16 ± 0.23	5
19	3-Me	1.2 ± 0.2	6.7
20	2-Cl	5.7 ± 1.3	10
2	2-CF3	0.8 ± 0.05	14

An X-ray crystal structure was determined for the complex of hBCATc with inhibitor **2** (Fig. 2). The ligand sits immediately adjacent to the PLP with the linker forming several hydrogen bonds: the sulfonyl and carbonyl oxygens with Ala 332 and both hydrozide NH's with Thr 258. Substituents at position 4 of ring A encounter steric interference from Tyr 88. Position 3 tolerates small substituents, probably with some rotation of the A-ring. Substituents at position 2 are not sterically restricted. Fluorines of the 2-CF_3 substituent lie 3.1 Å from the phenoxyl oxygen of Tyr 159, which accept a hydrogen bond from Arg 161 (not shown). A hydrogen bond to the CF_3 fluorine may stabilize the complex. The B ring lies against a hydrophobic side of the pocket and mainly interacts with Met 259. The furanyl oxygen may accept a hydrogen bond from the thiol of Cys 333. The 5-chloro

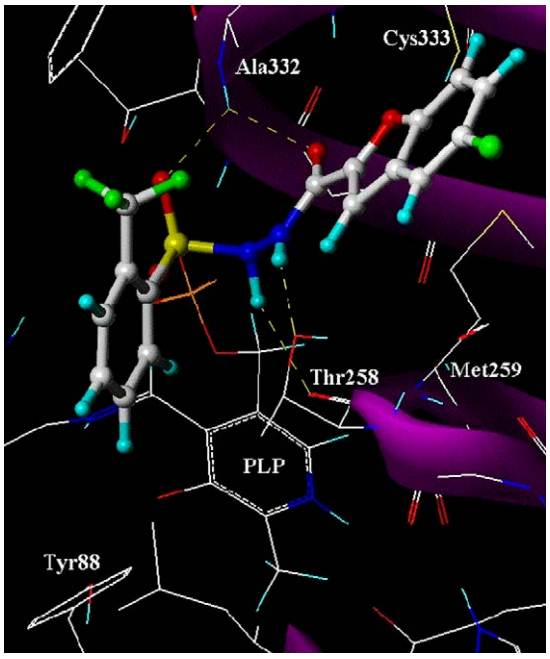


Figure 2. X-ray crystal structure of compound **2** bound to human cytosolic BCAT.[10b]

substituent contacts an amide NH from Gln 242 (not shown).

Inhibitor **2** showed a favorable in vitro profile (potency, selectivity). To insure that the compound's selectivity for the cystosolic enzyme in rat, inhibitor **2** was tested in a recombinant rat BCATc assay and a crude rat BCATm[5] assay. The IC_{50} at rat BCATc was 0.2 μM ± 0.02 ($n = 2$) vs 3.0 μM ± 0.5 ($n = 5$) at rat BCATm. This selectivity of approximately 15-fold justified further evaluation in in vitro and in vivo assays. Inhibitor **2** was tested in a neuronal cell culture model, which measures ^{45}[Ca] influx into neurons following treatment with the glutamate uptake inhibitor, 1-pyrrolidine dicarboxylic acid. This assay was specifically developed to evaluate BCATc inhibitors assuming that reducing the source of glutamate would reduce the downstream effects of glutamate, such as calcium influx and cell death[6]. Compound **2** decreased calcium influx in neuronal cultures with an $IC_{50} = 4.8 \pm 1.2$ μM ($n = 4$).

Following a 30 mg/kg subcutaneous injection to Lewis rats, inhibitor **2** reached a peak plasma concentration (C_{max}) of 8.28 μg/ml at 0.5 h (t_{max}). The mean plasma exposure (AUC) value was 19.9 μg h/ml, and the mean terminal half-life ranged from 12 to 15 h, indicating favorable PK parameters of inhibitor **2**. Since BCATc is involved in the synthesis of glutamate, a model of slow neurodegeneration was selected for the in vivo evaluation of inhibitor **2**. Daily administration of the mitochondrial neurotoxin, 3-nitroproprionic acid[7] (3-NP), has been shown to produce striatal lesions, which leads to motor deficits. Figures 3A and B illustrate the deficits in rotorod and beam walking performance and demonstrate that inhibitor **2** (administered for 9 days) was able to almost completely reverse the effects of 3-NP[8]. In addition, histological examination of the brains from these animals demonstrated significant reduction in the number of sections with degenerating neurons using a silver degeneration stain[11] (Fig. 4).

In summary, a series of potent and selective inhibitors of BCATc has been developed and the X-ray crystal structure with inhibitor **2** bound to BCATc was obtained. Using inhibitor **2** as a prototype, neuroprotection has

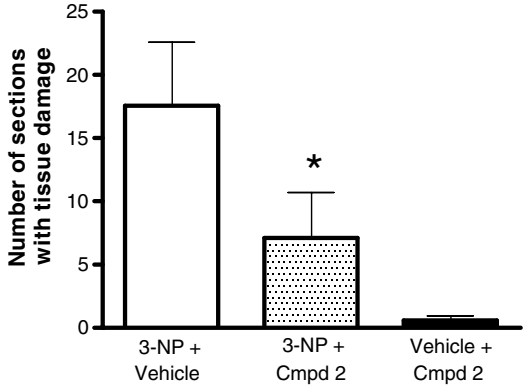


Figure 4. Histology following 3-NP administration and inhibitor **2.** Asterisks indicate $p < 0.05$ versus 3-NP plus vehicle treatment.

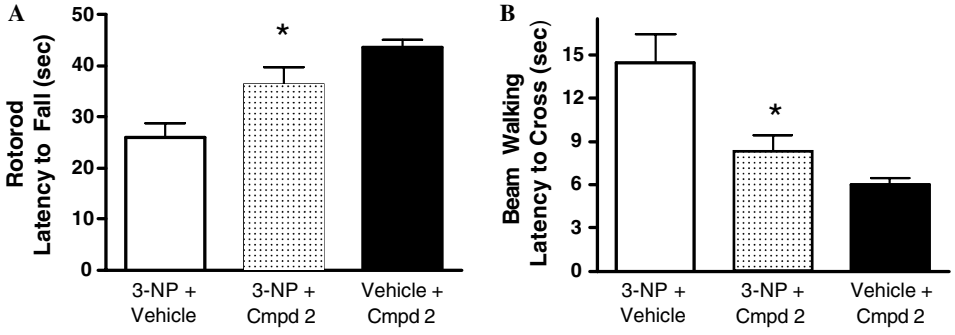


Figure 3. Effect of Inhibitor **2** on (A) rotorod performance and (B) beam walking in animals treated for 9 days with the 3-NP. Means ± SEM. Asterisks indicate $p < 0.05$ versus 3-NP plus vehicle treatment.

been demonstrated both in vitro and in vivo. This suggests that inhibitors of BCATc may represent a novel therapeutic strategy for the treatment of neurological disorders, where glutamate toxicity has been implicated.

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4. (a) Determination of IC_{50} values for BCAT inhibitors. The reaction mixtures (200 μl) contained 25 mM NaPi (pH 7.8), 0.75 mM L-leucine, 0.5 mM α-ketoglutarate, 10 μM pyridoxal phosphate, 1 mM NAD^+, 4 mM ADP, 2 mM dithiothreitol, 12.5 mM EDTA, the indicated compound, saturating level of glutamate dehydrogenase (GDH), and 50–100 nM hBCATc. The final concentration of DMSO was kept at 1%. The reactions were carried out in 96-well clear polystyrene plates. The reaction was incubated at 37 °C and the absorbance at 340 nm was collected over a period of 20 min with readings using a SpectraMax Microplate Spectrophoto-meter® (Molecular Devices, Palo Alto, CA) equipped with Softmax Pro® software (Molecular Devices, Palo Alto, CA). IC_{50}s were calculated from 8 to 10 point curves. Human BCATc was expressed in *Escherichia coli* and purified according to the method described.[1c] Glutamate dehydrogenase from bovine liver (Sigma) was purified further, as previously described.[4b] The amount of GDH was determined for each preparation to ensure the saturating level for the assay; (b) O'Carra, P.; Griffin, T.; O'Flaherty, M.; Kelly, N.; Mulcahy, P. *Biochim. Biophys. Acta* **1996**, *1297*, 235.
5. Inhibitors were solubilized in DMSO at a primary concentration of 10 mM and then diluted to give a final concentration between 0.3 and 100 μM. The assay is done in a 96-well plate. Total reaction volume of 100 μl consisted of 58 μl reaction mixture with 2μl of specified inhibitor and 38 μl water. The reaction was started with 2 μl BCAT enzyme. The 96-well reaction plate was incubated for 30 min at 37 °C. The reaction was stopped with 100μl of 2% acetic acid (1% Final) and analyzed for glutamate with LC/MS/MS.
6. (a) Cultures were prepared, as described in Ref. 6b. Ten- to fourteen-day-old cerebrocortical cultures (48-well plates) were washed once with 500 ml of 37 °C HBSS and then plated with 500 μl of 37 °C supplemented MEM (see formula below). A 2 μl volume of test agents (in 100% DMSO) was then spiked in at 260 times final desired concentrations. Plates were stored in a humidified CO_2 incubator at 37 °C for 2 h by which time 30 μCi/ml ^{45}Ca in MEM [0.60 μCi/ml final concentration] was added. Plates were then stored for one additional hour in a humidified CO_2 incubator at 37 °C and then spiked with 10 μl of either dH2O or 26 mM L-*trans*-pyrrolidine-2,4-dicarboxylate (LPDC) [500 μM final concentration]. Plates were then store in a humidified CO_2 incubator at 37 °C. ^{45}Ca uptake was determined thirty minutes following the addition of LPDC. Cultures were washed trice with 500 ml HBSS and then dissolved with lysate buffer. Individual lysates were combined with 10 ml scintillation cocktail and counted for emissions using a Tricarb 2900TR Packard Liquid Scintillation Analyzer; (b) Marcoux, F. W.; Probert, A. W. *Stroke* **1988**, *19*, 148.
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8. The study began with 3 days of dosing with compound **2** at 30 mg/kg, SC, BID (60 mg/kg/day) and continued daily for 14 days. The mitochondrial inhibitor 3-nitropropionic acid (3-NP, 46 mg/kg, IP) was administered every other day up to day 9 (day 1 defined by the first day of 3-NP dosing). Behavioral testing was done at baseline, during 3-NP administration (day 6), at day 14 (3 days after the end of compound **2** dosing), and again at day 20. Data in Figure 3 represent the maximum effect seen on two of the motor function endpoints. As evaluated by ANOVA, there was an overall statistical difference for each of the behavioral endpoints, and Bonferroni corrected post hoc *t*-tests were used for specific comparisons. After dosing and behavioral tests, the rats were cardiac-perfused, washed with cold saline, and brains were removed. The right hemispheres were immediately immersed into 4% paraformaldehyde and kept under 4 °C for at least a week. The brain samples were then paraffin embedded and continuous brain sections (40 μm in thickness) were obtained from olfactory bulb to the end of occipital cortex. Every 8th section was processed for cupric silver staining that demonstrates degenerative areas.[11] For evaluation of brain damage, sections were examined with a light microscope for detecting positive staining black areas that implied areas of neuronal death. Total number of sections with the positive findings from each group was finally compared with other groups (Newman–Keuls).
9. (a) All the compounds were synthesized by coupling the aryl sulfonyl chloride and aryl carboxylic acid hydrazide in pyridine, as described in compound **2**. Compound **2**: A solution of 2-trifluorophenyl sulfonyl chloride (1 mmol) and 5-chloro-benzofuran-2-carboxy-lic acid hydrazide (1 mmol) was stirred for 18 h at room temperature in pyridine (10 ml). After the pyridine was removed in vacuo, the residue was washed with water and purified by column over SiO_2 eluting with 1:2 EtOAc/hexanes (60% yield). MS: 419.9 (M + 1 for $C_{16}H_{10}N_2O_4SF_3Cl$); mp: 192–193.4 °C. Analysis ($C_{16}H_{10}N_2O_4SF_3Cl$) calcd. C: 45.89, H: 2.41, N: 6.69, S: 7.66, F: 13.61. Found: C: 46.16, H: 2.41, N: 6.60, S: 7.38, F: 13.30. ^1H NMR (DMSO-d_6) δ 7.44 (dd, 1H, J = 9.04 and 2.20 Hz), 7.53 (s, 1H), 7.63 (d, 1H, J = 8.79 Hz), 7.75–7.82 (m, 2H), 7.84 (d, 1H, J = 1.95 Hz), 7.93 (dd, 1H, J = 2.2 and 9.03 Hz), 8.13 (dd, 1H, J = 2.44 and 6.59 Hz), 10.19 (s, 1H), and 11.06 (s, 1H); (b) Hu, L.-Y.; Kesten, S.R.; Lei, H.; Wustrow, D.J.; Ryder, T.R.E.P. EP 1,314,723, 2003.
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