Supporting information

Discovery of the First Selective Inhibitor of Excitatory Amino Acid Transporter Subtype 1

By

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Chemistry

Chemistry. All reagents were obtained from commercial suppliers and used without further purification. The microwave reactions were carried out in a Biotage Initiator with autosampler (0-300 W at 2.45 GHz). Flash chromatography (FC) was performed using CombiFlash Companion System (Teledyne Isco, Inc.) on Redi*Sep* columns with silica gel (average particle size 35-70 microns). Vacuum liquid chromatography (VLC) was performed using silica gel (average particle size 15-40 microns). NMR (300 MHz) spectra were recorded in DMSO-D6 using DMSO as reference, unless otherwise noted. Melting points are uncorrected.

2-Amino-4-(4-methoxyphenyl)-7-(phenyl)-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (1c). Equimolar amounts of 5-phenylcyclohexane-1,3-dione (0.095 g, 0.5 mmol), 4-methoxybenzaldehyde (61 µL, 0.5 mmol) and malononitrile (0.034 g, 0.5 mmol) were mixed together in H_2O/EtOH (1:1, 2 mL), and were allowed to react under microwave irradiation (reaction time: 30 min., temp.: 85°C, pre-stirring: 3 min., vial size: 2-5 mL, absorption level: high, fixed hold time: on). The reaction mixture was allowed to cool to rt, the solvent was filtered off and the solid obtained was washed with H_2O. Yield: 0.156 g (84 %) white crystalline powder. Mp: 194.4-206.7°C (decompose). ^{1}H NMR δ: 7.38-7.18 (m, 5H), 7.11 (d, 1H), 7.03-6.93 (m, 3H), 6.84 (d, 1H), 6.78 (d, 1H), 4.17 (s, 0.5H), 4.16 (s, 0.5H), 3.71 (s, 1.5H), 3.70 (s, 1.5H), 3.57-3.34 (m, 1H), 3.08-2.88 (m, 1H), 2.78-2.58 (m, 2H), 2.47-2.34 (m, 1H). ^{13}C NMR δ: 195.9, 164.4, 163.6, 159.3, 159.1, 158.8, 158.7, 143.5, 137.7, 137.4, 129.3, 129.1, 127.8, 120.7, 114.8, 114.6, 114.4, 59.3, 55.9, 44.2, 38.5, 38.2, 35.7, 35.5, 34.6, 34.5.

2-Amino-4-(4-methoxyphenyl)-7-(3,4-dimethoxyphenyl)-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (1n). The product was prepared according to reference 1. The product, **1n,** was isolated as a pale yellow powder: mp = 159.9-161.4 °C. ^{1}H NMR δ: 7.23-6.69 (m, 7H), 4.37 (br t, 0.5H), 4.19 (br s, 0.5H), 3.72 (br s, 6H), 3.36 (s, 3H), 3.31-3.16 (m, 1H), 3.12-2.84 (m, 1H), 2.84-2.56 (m, 1H), 2.51 (br s, 2H), 2.84-2.26 (m, 1H). ^{13}C NMR δ: 195.0, 195.0, 163.5, 162.6, 158.3, 158.2, 157.8, 157.7, 148.5, 147.42, 147.39, 136.7, 136.5, 135.1, 135.0, 128.2, 119.7, 118.6, 118.3, 113.8, 113.6, 113.5, 111.7, 111.5, 111.0, 110.7, 58.4, 58.3, 56.0, 55.5, 55.4, 55.36, 55.04, 55.00, 43.7, 37.5, 37.1, 34.8, 34.7, 34.1, 34.0.

2-Amino-4-(4-methoxyphenyl)-7-(naphthalene-1-yl)-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (1o). 4-Methoxybenzaldehyde (0.102 mL, 0.839 mmol) was added to a mixture of 5-(naphthalen-1-yl)cyclohexane-1,3-dione (0.204, 0.854 mmol), and malononitrile (0.0593 g, 8.98 mmol) in 99.9 % ethanol (1.5 mL). 4-Methylmorpholine (0.093 mL, 0.844 mmol) was added and the mixture was stirred for 10 min. after which it was left to stand at room temperature for 17 h followed by 4½ h at 5 °C. The fluid above the precipitate was decanted and left to stand at room temperature for 20 h. The precipitated crystals was filtered off and washed with ethanol. Yield: 0.222 g (63 %) white crystals. Mp: 194.0-197.2 °C. ^{1}H NMR δ: 8.20 (d, 1H), 7.92 (d, 1H), 7.81 (d, 1H), 7.61-7.47 (m, 2H), 7.44-7.35 (m, 1H), 7.35-7.28 (m, 1H), 7.05 (d, 2H), 6.98 (br s, 2H), 6.82 (d, 2H), 4.47-4.33 (m, 1H), 4.22 (s, 1H), 3.72 (s, 3H), 3.14-2.87 (m, 2H), 2.83-2.58 (m, 2H). ^{13}C-NMR δ: 195.8, 163.4, 159.0, 158.5, 138.9, 137.2, 134.2, 131.2, 129.5, 129.0, 128.0, 127.1, 126.4, 126.1, 124.1, 123.7, 120.6, 114.29, 114.26, 59.2, 55.8, 43.8, 35.8, 34.2, 33.7.

2-Amino-5-oxo-7-phenyl-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (1w). Formaldehyde, 37 wt.% solution in water (0.126 mL, 4.5 mmol) was added to a mixture of 5-phenylcyclohexane-1,3-dione (0.860, 4.6 mmol) and malononitrile (0.300 g, 4.5 mmol) in 99.9 % ethanol (5.0 mL). 4-methylmorpholine (0.50 mL,

4.5 mmol) was added and the mixture was stirred for 10 min. after with it was left to stand at 5 °C for 20 h. The precipitate was filtered off and washed with ice cold ethanol and ice cold heptane. Recrystallized from propan-2-ol, then purified by VLC on a silica gel column with 5:1 heptane/EtOAc. Yield: 0.105 g (9 %) pale yellow crystals. Mp: 178.5-181.5 °C. R_f(1:1 heptane/EtOAc): 0.42. ^{1}H NMR (CD_3CN) δ: 7.39-7.22 (m, 5H), 4.51 (br s, 2H), 3.47-3.36 (m, 1H), 2.99 (s, 2H), 2.81-2.60 (m, 4H). ^{13}C NMR ($CDCl_3$) δ: 196.1, 163.0, 158.1, 141.8, 129.0, 127.4, 126.6, 119.4, 111.1, 56.3, 43.7, 38.7, 34.6, 18.8.

(*E*)-4-(naphthalene-1-yl)but-3-en-2-one. To a suspension of sodium hydride 60 % dispersion in mineral oil (0.560 g, 14.0 mmol) in dry diethyl ether (44 mL) was added a solution of diethyl 2-oxopropylphosphonate (2.8 mL, 14.6 mmol) in dry diethyl ether (10 mL). The solution was stirred for 1½ h at room temperature after which a solution of 1-naphthaldehyde (1.8 mL, 13.2 mmol) in dry diethyl ether (5 mL) was added. After stirring for 69 h at room temperature saturated $NH_4Cl_{(aq)}$ (20 mL) was added to the solution whereby all precipitated material dissolved. The aqueous layer was extracted with diethyl ether (3 x 20 mL), dried ($MgSO_4$), filtrated and evaporated *in vacuo* to give an orange oil. Purified by VLC on a silica gel column with 10:1 heptane/EtOAc. Yield: 2.23 g (89 %) yellow oil. R_f(10:1 heptane/EtOAc): 0.15. ^{1}H NMR ($CDCl_3$) δ: 8.38 (d, 1H), 8.16 (d, 1H), 7.90 (t, 2H), 7.77 (d, 1H), 7.62-7.47 (m, 3H), 6.82 (d, 1H), 2.48 (s, 3H). ^{13}C-NMR ($CDCl_3$) δ: 198.3, 140.2, 133.7, 131.7, 131.5, 130.8, 129.6, 128.9, 127.0, 126.3, 125.6, 125.2, 123.2, 28.1.

5-(naphthalene-1-yl)cyclohexane-1,3-dione. To a solution of sodium ethoxide 21 % in ethanol (2.6 mL, 6.91 mmol) was added diethyl malonate (1.1 mL, 6.91 mmol) followed by (*E*)-4-(naphthalene-1-yl)but-3-en-2-one (1.356 g, 6.91 mmol) dissolved in dry 99.9 % ethanol (3 mL). The reaction mixture was refluxed for 6 h, and then concentrated *in vacuo* to give a solid. The solid was suspended in water (70 mL), saturated aq. NaCl (20 mL) was added and the aqueous suspension was washed with dichloromethane (3x100 mL). The aqueous layer was concentrated *in vacuo* to a crude solid. 2 M NaOH (10 mL) was added, and the reaction mixture was refluxed for 2 h. To the cooled reaction mixture was added 2 M H_2SO_4 (12.5 mL) and the reaction mixture was refluxed for 4.5 h after which it was allowed to cool to room temperature over night. The cooled reaction mixture was extracted with dichloromethane (5x50mL). The organic layer was evaporated *in vacuo* to give a pale yellow solid. Purified by FC using 60:40:2:1 heptane/EtOAc/ MeOH/AcOH. Yield: 0.537 g (33 %) white crystalline solid. R_f(60:40:2:1 heptane/EtOAc/MeOH/AcOH): 0.15. ^{1}H NMR δ: 11.21 (br s, 1H), 8.16-8.14 (m, 1H), 7.95-7.92 (m, 1H), 7.82-7.79 (m, 1H), 7.58-7.45 (m, 4H), 5.34 (s, 1H), 4.24-4.13 (m, 1H), 2.88-2.43 (m, 4H). ^{13}C-NMR δ: 139.7, 134.0, 131.2, 129.4, 127.6, 126.9, 126.2, 126.1, 123.7, 123.5, 104.1, 40.4, 40.2, 34.4 (C=O signals not observed even with relaxation time d1=100).

Table 1. Elemental analysis (CHN) of key target compounds

	Brutto formula	**CHN Calculated**	**CHN Found**
1c	$C_{23}H_{20}N_2O_3$	74.18; 5.41; 7.52	74.06; 5.36; 7.30
1n	$C_{25}H_{24}N_2O_5$	69.43; 5.59; 6.48	--
	$C_{25}H_{24}N_2O_5$*0.75(EtOH)*0.5(H_2O)	66.86; 6.25; 5.88	66.92; 5.83; 5.48
1o	$C_{27}H_{22}N_2O_3$	76.76; 5.25; 6.63	--
	$C_{27}H_{22}N_2O_3$*0.33(EtOH)	75.90; 5.53; 6.40	75.59; 5.20; 6.65
1w	$C_{16}H_{14}N_2O_2$	72.16; 5.30; 10.52	71.88; 5.17; 10.42

Pharmacology

Materials. The cDNAs for the human EAATs and the rat EAATs were kind gifts from Drs. Susan Amara (University of Pittsburgh School of Medicine, Pittsburgh, PA) and Thomas Rauen (University of Osnabrück, Germany), respectively. Culture medium, serum and antibiotics for cell culture were obtained from Invitrogen (Groningen, The Netherlands), [^{3}H]-D-Aspartate ([^{3}H]-D-Asp) was obtained from Amersham (Buckinghamshire, UK), and Glu and DL-TBOA were purchased from Sigma (St. Louis, MO) and Tocris Cookson (Bristol, UK), respectively. The library of 3,040 compounds was purchased from Chembridge Corporation (San Diego, CA). Analogs **1c**, **1n**, **1o**, **and 1w** were synthesized by us (see chemistry section) whereas the remaining 21 analogs were purchased from Chembridge Corporation or other commercial sources. All 25 analogs are characterized as a mixture of stereoisomers (ratios of isomers were not determined).

Cell culture and transfections. The cell lines used in this study were maintained at 37 °C in a humidified 5% CO_2 incubator. The construction of stable HEK293 cell lines expressing the human EAAT subtypes EAAT1, EAAT2 and EAAT3 has been described previously [2]. These cell lines were grown in culture medium [Dulbecco's Modified Eagle Medium (DMEM) supplemented with penicillin (100 U/mL), streptomycin (100 □g/ml) and 5% dialyzed fetal bovine serum] supplemented with 1 mg/ml G-418. The tsA-201 cells used for the transient transfections with rat EAAT cDNAs were grown in culture medium and transfected with GLAST-pBK/CMV, GLT-1-pBK/CMV or EAAC-1-pBK/CMV using Polyfect as a DNA carrier according to the protocol by the manufaturer (Qiagen, Hilden, Germany).

[^{3}H]-D-Asp uptake assay. The screening of the compound library of 3,040 compounds and the subsequent characterization of compounds **1a-y** at the EAAT1-, EAAT2- and EAAT3-HEK293 cell lines was performed in a [^{3}H]-D-Asp uptake assay essentially as previously described.[2] Briefly, cells were split into poly-D-lysine-coated white 96-well plates (PerkinElmer, Boston, MA). 16-24 h later the culture medium was aspirated, and cells were washed twice with 100 µL assay buffer (Phosphate Buffered Solution supplemented with 2 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4). Then 50 µL assay buffer supplemented with 30 nM [^{3}H]-D-Asp and the various compounds were added to the various wells, and the plate was incubated at 37 °C for 7 min. The assay mixtures was quickly removed from the wells, which were then washed with 3 x 100 µL ice-cold assay buffer, and 150 µL Microscint^{TM}20 scintillation fluid (PerkinElmer, Boston, MA) was added to each well. The plate was shaken for 1 hour and counted in a TopCounter (PerkinElmer, Boston, MA). The experiments were performed in duplicate 3-7 times for each compound. Concentration-inhibition curves were generated by nonweighted least-squares fits using the program KaleidaGraph 3.6 (Synergy Software, Reading, PA).

The FLIPR® Membrane Potential Blue assay. The pharmacological properties of Glu, compound **1o** and DL-TBOA at the stable EAAT1-, EAAT2- and EAAT3-HEK293 cell lines and at rat GLAST, GLT1 or EAAC1 transiently expressed in tsA-201 cells were determined in the FLIPR® Membrane Potential (FMP) Blue assay (Molecular Devices, Crawley, UK) essentially as previously described.[2] Briefly, cells were split into poly-D-lysine-coated black clear bottom 96-well plates (BD Biosciences, Bedford, MA). 16-24 h later the culture medium was aspirated, and the cells were washed with 100 µl Krebs buffer [140 mM NaCl/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgCl_2$/11 mM HEPES/10 mM D-Glucose, pH 7.4]. Then 50 µL Krebs buffer was added to each well (in the experiments with **1o** and DL-TBOA, the compounds were added to this buffer). 50 µL

Krebs buffer supplemented with FMP assay dye (1 mg/mL) was then added to each well, and the plate was incubated at 37 °C for 30 min. The plate was assayed at room temperature in a NOVOstar™ plate reader (BMG Labtechnologies, Offenburg, Germany) measuring emission at 560 nm caused by excitation at 530 nm before and up to 1 min after addition of 33 µl Glu solution (dissolved in Krebs buffer). Since K_m values for Glu at EAAT1, EAAT2, EAAT3, GLAST, GLT-1 and EAAC-1 ranged from 16 to 45 mM (see Results section), a final assay concentration of 50 µM Glu was used for all the transporters for the testing of the inhibitors. The experiments were performed in duplicate 3-4 times for each compound. The concentration-response curves for Glu and concentration-inhibition curves for **1o** and DL-TBOA were constructed based on the maximal responses obtained for 8 different concentrations of each of the compounds and generated by nonweighted least-squares fits using the program KaleidaGraph 3.6 (Synergy Software, Reading, PA).

References

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