First Demonstration of a Functional Role for CNS Betaine/GABA Transporter (mGAT2) Based on Synergistic Anticonvulsant Action Among Inhibitors of mGAT1 and mGAT2

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a) Running Title: Function and Pharmacology of CNS Betaine/GABA Transporter

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c) Number of text pages: 26
Number of tables: 4
Number of figures: 5
Number of references: 40
Number of words in Abstract: 240
Number of words in Introduction: 712
Number of words in Discussion: 1467

d) List of nonstandard abbreviations
CI-966, N-2- bis[4-(trifluoromethylphenyl) methoxy]ethyl guvacine
CNS, central nervous system
EF1500, N-[4,4-bis (3-methyl-2-thienyl)-3-butenyl]-3-hydroxy-4-amino-4,5,6,7-
tetrahydrobenzo[d]isoxazol-3-ol
EF1502, N-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-hydroxy-4-(methylamino)-4,5,6,7-tetrahydrobenzo[d]isoxazol-3-ol
exo-THPO, 4-amino-4,5,6,7-tetrahydrobenzo[d]isoxazol-3-ol
GABA, gamma aminobutyric acid
GAT, gamma aminobutyric acid transporter
HEK, human embryonic kidney
LU-32-176B, N-[4,4-bis(4-fluorophenyl)-butyl]-3-hydroxy-4-amino-4,5,6,7-
tetrahydrobenzo[d]isoxazol-3-ol
PTZ, pentylenetetrazol
SKF 89976A, (R)-N-(4,4-diphenyl-3-butenyl)nipecotic acid
SKF 100330A, N-(4,4-diphenyl-3-butenyl)guvacine

e) Recommended section assignment: Neuropharmacology
Abstract

In a recent study, EF1502 (N-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-hydroxy-4-(methylamino)-4,5,6,7-tetrahydrobenzo[d]isoxazol-3-ol) which is an N-substituted analog of the GAT1 selective GABA uptake inhibitor exo-THPO (4-amino-4,5,6,7-tetrahydrobenzo[d]isoxazol-3-ol) was found to inhibit GABA transport mediated by both GAT1 and GAT2 in human embryonic kidney (HEK) cells expressing the mouse GABA transporters GAT1-4 (mGAT1-4). In the present study, EF1502 was found to possess a broad-spectrum anticonvulsant profile in animal models of generalized and partial epilepsy. When EF1502 was tested in combination with the clinically effective GAT1 selective inhibitor tiagabine ((R)-N-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]nipecotic acid) or LU-32-176B (N-[4,4-bis(4-fluorophenyl)-butyl]-3-hydroxy-4-amino-4,5,6,7-tetrahydrobenzo[d]isoxazol-3-ol), another GAT1 selective N-substituted analog of exo-THPO, a ‘synergistic’ rather than ‘additive’ anticonvulsant interaction was observed in the Frings audiogenic seizure-susceptible mouse and the pentylenetetrazol seizure threshold test. In contrast, combination of the two mGAT1-selective inhibitors tiagabine and LU-32-176B resulted in only an ‘additive’ anticonvulsant effect. Importantly, the combination of EF1502 and tiagabine did not result in a greater than additive effect in the rotarod behavioral impairment test. In subsequent in vitro studies conducted in HEK-293 cells expressing the cloned mouse GAT transporters mGAT1 and mGAT2, EF1502 was found to non-competitively inhibit both mGAT1 and the betaine/GABA transporter mGAT2 (K_i's: 4 and 5 µM, respectively). Furthermore, in a GABA release study conducted in neocortical neurons EF1502 did not act as a substrate for the GABA carrier. Collectively, these findings support a functional role for mGAT2 in the control of
neuronal excitability and suggest a possible utility for mGAT2 selective inhibitors in the treatment of epilepsy.
Reduction of GABA-mediated inhibitory neurotransmission is associated with seizure activity and drugs which elevate synaptic GABA levels either by inhibition of GABA degradation or inhibition of high affinity transport have been demonstrated to possess anticonvulsant activity (see Dalby, 2003; Sarup et al., 2003). For example, the GABA-transaminase inhibitor vigabatrin and the GABA-transport inhibitor tiagabine are clinically effective antiepileptic drugs (see Ben-Menachem, 2002; Kalviainen, 2002, for review and references).

Since the advent of cloning of several GABA transporters from different species including mouse, rat, and human, interest has been focused on the development of inhibitors of the GABA transporter-1 (GAT1). In support of this principle, a number of GAT1-selective inhibitors have been developed and demonstrated to possess anticonvulsant activity in animal seizure models (Swinyard et al., 1991; Pavia et al., 1992; Suzdak and Jansen, 1995; White et al., 2002; Dalby, 2003). For example, the first peripherally active GABA uptake inhibitor was SKF 89976A ((R)-N-(4,4-diphenyl-3-butenyl) nipecotic acid), an N-substituted diphenyl butenyl analog of nipecotic acid (Swinyard et al., 1991). The corresponding analogs of guvacine, SKF 100330A (N-(4,4-diphenyl-3-butenyl)guvacine) and CI-966 (N-2- bis[4-(trifluoromethyl)phenyl] methoxy)ethyl guvacine), supported the principle that modulation of GABA transport would result in an anticonvulsant action (Swinyard et al., 1991; Pavia et al., 1992). Unfortunately subsequent clinical trials with CI-966 revealed the potential for serious psychotic adverse effects in certain patients (Radulovic et al., 1993).
Tiagabine ((R)-N-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]nipecotic acid) is a second-generation analog of nipecotic acid that contains the dithienyl ring system in place of the diphenyl rings and was developed clinically without any obvious psychotropic adverse effects. To date, tiagabine is the only clinically available GABA transport inhibitor used for the treatment of epilepsy. However, since its introduction into the world-wide market, a number of individual case reports have suggested that tiagabine, if not titrated correctly or employed in patients with pre-existing psychiatric disorders, may possess a similar adverse-effect profile (Trimble et al., 2000) as that predicted by for CI-966 (Radulovic et al., 1993). Although acute psychosis has not been substantiated in more recent studies (Sackellares et al., 2002), tiagabine use has been associated with an increased incidence of depression (see Kalviainen, 2002, for review). These patient reports demonstrate the continued need for less toxic third-generation GABA transport inhibitors.

In this regard, it would seem advantageous if such transport inhibitors would exhibit an inhibition profile different from that of tiagabine, i.e., an affinity for transporters other than GAT1 such as GAT2-4. To this end, Dalby et al. (1997) have demonstrated that such inhibitors may be interesting as antiepileptic drugs. For example, it was reported that inhibitors of GAT3 and GAT4 (Thomsen et al., 1997) afforded protection against maximal electroshock-, audiogenic-, and kindled-seizures (Dalby et al., 1997) Unfortunately these inhibitors, which structurally are not GABA mimetics (Thomsen et al., 1997), were shown to have affinity for α-1 adrenergic and D-2 dopamine receptors (Dalby et al., 1997). As such, it is difficult to make any definitive conclusion regarding the mechanism underlying their anticonvulsant action.
The present study was undertaken in an effort to obtain detailed information about newly developed N-substituted analogs of exo-THPO (4-amino-4,5,6,7-tetrahydrobenzo[d]isoxazol-3-ol; see Figure 1) which have been shown to inhibit to varying degrees GABA transporters other than GAT1 (Clausen et al., 2005). Of particular interest is the analog EF1502 (N-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-4-(methylamino)-4,5,6,7-tetrahydrobenzo[d]isoxazol-3-ol) which was found to equipotently inhibit both mGAT1 and mGAT2 and was completely devoid of activity at a multitude of neurotransmitter systems including adrenergic, GABAergic, and dopaminergic receptors (radioligand binding study of 74 different receptors and ion channels; data not shown). The role of mGAT2 within the brain is poorly understood. In addition to transporting GABA, this transporter is thought to regulate the uptake of the osmo-regulator betaine (see Borden et al., 1995 for review and references). However, it is not clear whether modulation of mGAT2 contributes to control of neuronal excitability. As such, the present investigation employed a number of animal seizure and epilepsy models to characterize the anticonvulsant profile of this particular exo-THPO analogs and to elucidate a functional role for the betaine/GABA transporter mGAT2. The findings obtained from isobolographic analysis of results obtained from combination studies conducted with EF1502 and tiagabine or LU-32-176B (N-[4,4-bis(4-fluorophenyl)-butyl]-3-hydroxy-4-amino-4,5,6,7-tetrahydrobenzo[d]isoxazol-3-ol) suggests a functional role for mGAT2 in the control of seizure activity. These findings provide the basis for this report.
Methods

Materials

Plastic tissue culture dishes were purchased from NUNC Denmark, and fetal calf serum from Sera-Lab, Sussex, UK. Poly-D-lysine (molecular weight >300,000), trypsin, soybean trypsin inhibitor, DNAse, cytosine arabinoside and amino acids were obtained from Sigma (St. Louis, MO, USA); insulin from NOVO-Nordisk A/S, (Bagsvaerd, Denmark); and penicillin from Leo Pharma (Ballerup, Denmark). [3H]GABA (79.0 Ci mmol\(^{-1}\)) was from Dupont-NEN (Frankfurt, Germany). Exo-THPO and its N-substituted analogs were synthesized as previously described (Clausen et al., 2005). Tiagabine was generously provided by Sanofi Synthelabo (Brøndby, Denmark). All other chemicals were of the purest grade available from regular commercial sources. The accession numbers for mouse GAT1 and GAT2 are M92378 and M97632, respectively.

Cultured Neurons

Cerebral cortical neurons were isolated and cultured from 15-day-old mouse embryos which were obtained from Bomholtgaard (Ry, Denmark). After dissociation of the tissue by trypsinization and trituration in a DNAse solution containing soybean trypsin inhibitor as described by Hertz et al. (1989), the cells were plated onto NUNC 35 mm Petri dishes. After 48 h in culture, 20 µM (final concentration) cytosine arabinoside was added to the culture medium to prevent astrocytic proliferation (Larsson et al., 1985). Cells were cultured for 7-8 days at which time the neurons had become functionally differentiated with properties resembling mature GABAergic neurons such as high
activity of glutamate decarboxylase and pronounced vesicular GABA release (Hertz and Schousboe, 1987).

Subcloning and Expression of GABA Transporters

The cDNAs encoding the two murine GABA transporters GAT1 and GAT2 (mGAT1 and mGAT2, respectively; Liu et al., 1992; Lopez-Corcuera et al., 1992; Liu et al., 1993) were subcloned into the mammalian expression vector, pCis as previously described (Bolvig et al., 1999). GAT-1pBSK was digested with XbaI, blunt-ended with Klenow enzyme, and subsequently digested with Nhel. The 2.2 kbp fragment containing GAT-1 was then ligated into the 5'-ClaI (blunt-ended with Klenow enzyme) and 3'-XbaI sites of pCis vector. The 1.9 kbp XbaI (5')-Nhel (3') GAT-2 fragment from GAT-2pBSK was ligated into the XbaI site of pCis vector. GATpCis cDNAs were transformed into XL1-Blues bacteria (Stratagene, La Jolla, CA) and plasmids prepared by Qiagen (Chatsworth, CA) column purification. Human embryonic kidney (HEK)-293 cells were maintained in complete growth medium (MEM with Earle's salts, supplemented with 5% fetal calf serum, 1% Anti-PPLO and 1% Glutamax I, pH 7.3 under 5% CO₂). GATpCis transfections were carried out as described previously (White et al., 2002).

[^3H]GABA Uptake

The uptake of[^3H]GABA in the recombinant cell systems was investigated essentially as described previously (Bolvig et al., 1999; White et al., 2002). The incubations, performed at 37°C in phosphate buffered saline, were initiated by exchanging the culture medium with incubation medium leaving the cells attached to the
bottom of the wells during the entire procedure and terminated (after 3 min) by rapid wash with non-radioactive incubation medium. In the kinetic experiments, the GABA concentration was varied over the range 1-1,000 µM. The concentrations of uptake inhibitors present during incubations in the kinetic assays are stated in Table 2. After incubation, the cells were dissolved in 0.4 M KOH and radioactivity (Schousboe et al., 1977) and protein concentrations (Lowry et al., 1951) were determined. Protein contents were related to bovine serum albumin used as the standard. The kinetic parameters, $V_{\text{max}}$ and $K_m$, of the carrier-mediated, high-affinity GABA uptake in HEK-293 cells expressing mGAT1 and mGAT2 were calculated (Larsson et al., 1986a,b) by means of a computer program for unconstrained minimization or via the computer program Grafit v3.0 (Erithacus Software, UK), fitting to the following equation: 

$$
V = \frac{V_{\text{max}} S}{(K_m + S)} + kS.
$$

The non-saturable influx component ($kS$) varied somewhat between batches but was in the range of $10^{-3}$-$10^{-2}$ ml min$^{-1}$ mg$^{-1}$. There was no systematic variation of this component depending on the presence or absence of inhibitors. In the case of non-competitive inhibition, the $K_i$ values were calculated from the determined $V_{\text{max}}$ values of the control- and test-situation using the following equation: 

$$
K_i = \frac{I}{[V_{\text{max}}/V'_{\text{max}} - 1]};
$$

where $I$ is the inhibitor concentration, $V_{\text{max}}$ is the control value, and $V'_{\text{max}}$ is the value determined in the presence of inhibitor.

**GABA Release**

Cerebral cortical neurons in culture were preloaded with [³H]-GABA (1 µM, 0.1 µCi) for 30min in the presence of 10 µM vigabatrin to irreversibly inactivate GABA-transaminase thereby blocking GABA metabolism (Drejer et al., 1987; Gram et al.,
1988). Subsequently, individual cultures (35 mm Petri dishes) were placed in a superfusion apparatus (Drejer et al., 1987) at 37°C equipped with peristaltic pumps and the cells superfused at a flow rate of 2 ml/min. Fractions from the outlet were collected every 30 sec and at the end of the experiments radioactivity was determined in all fractions. During the superfusion, either 200 µM of non-radioactive GABA or 200 µM EF1502 was added to the superfusion medium for two min. Results were expressed as cpm per fraction collected. It should be noted that since the baseline of the GABA release during the entire superfusion period was constant no major loss of intracellular [3H]GABA occurred during this period.

**Animals**

Male albino CF No 1 mice (18-25 g, Charles River, Wilmington, MA) and male and female Frings audiogenic seizure-susceptible mice (18-25 g, Anticonvulsant Drug Development Program, University of Utah, Salt Lake City, UT) were used as experimental animals for studies conducted at the University of Utah. For these studies, all animals were allowed free access to both food (S/L Custom Lab Diet-7) and water except when they were removed from their cages for the experimental procedure. Mice were housed in a temperature and humidity controlled facility and were maintained on 12 hour light-dark cycle beginning at 6:00 a.m. Furthermore, all mice were housed, fed, and handled in a manner consistent with the recommendations in HEW publication (NIH) No. 86-23, "Guide for the Care and Use of Laboratory Animals." Animals were euthanized in accordance with Public Health Service policies on the humane care of laboratory animals.
For those studies conducted at H. Lundbeck A/S, male Wistar rats (280-320 g at start of experiment) from M&B (Denmark) were used for amygdala kindling. For the i.v. PTZ and intraperitoneal (i.p.) pilocarpine tests, male NMRI mice (M&B, Denmark) 18-22 g were used. All animals were allowed to adapt to the laboratory environment for at least one week prior to testing. Animals were housed on a 12 hour light-dark cycle, beginning at 6:00 am. All experiments were conducted between 8:00 am and 2:00 pm. Experimental work was performed under a license from the Danish Ministry of Justice, and in accordance with EC Directive 86/609/EEC and the Danish law regulating experiments on animals.

The test substances were either suspended or dissolved in 0.5% methylcellulose by trituration in a mortar and pestle and sonicating for 10 min. All substances were administered i.p. or s.c. in a volume of 0.01 ml/g body weight in mice and 0.05 ml/g body weight in rats.

Anticonvulsant Testing

The anticonvulsant profile of EF1502 was established by several different anticonvulsant tests that included audiogenic-, six Hz psychomotor-, amygdala kindled-, PTZ-, and pilocarpine- seizures.

Audiogenic Seizures

The ability of LU-32-176B, EF1502, and tiagabine to prevent sound-induced seizures in the Frings AGS-susceptible mouse model was assessed following i.p. administration.
For each of the analogs examined, time-response curves were constructed using a dose that produced a submaximal anticonvulsant effect in a group of 4 mice per time point tested (5, 15, 30, 60, and 120 min). Dose-response studies were then conducted at the subsequently determined time of peak effect of each test substance. For these studies, individual mice were placed into a plexiglass cylinder (diameter, 15 cm; height 18 cm) fitted with an audio transducer (Model AS-ZC; FET Research and Development, Salt Lake City, UT) and exposed to a sound stimulus of 110 decibels (11 KHz) delivered for 20 sec. Sound-induced seizures are characterized by wild running followed by loss of righting reflex with forelimb and hindlimb tonic extension. Mice not displaying hindlimb tonic extension were considered protected.

The anticonvulsant activity for each of the test compounds was then quantitated in groups of 4-8 mice per dose level. Varying doses of each substance were administered to groups of mice until at least two points were established between the limits of 0 and 100% protection.

In addition to assessing the percent protection following exposure to audiogenic stimulation, the effect of each analog on the audiogenic seizure severity was determined by assigning a score to the seizure phenotype as follows: 0, no response; 1, wild running for < 10 sec; 2, wild running for > 10 sec; 3, all-limb clonus; 4, forelimb extension; 5, forelimb and hindlimb extension.

The dose of drug required to produce the desired endpoint in 50% of animals tested (i.e., the ED$_{50}$) and the 95% confidence interval were then calculated by Probit analysis of the dose-response data using a computer program based on the method described by Finney (1971).
EF1502 and LU-32-176B were also tested in combination with tiagabine in the Frings AGS mouse. For these studies, varying doses (administered as a fraction of their ED$_{50}$ value) of one of the drugs were administered i.p. The effect of a given dose-level on the efficacy of the second drug was then established by re-determining the ED$_{50}$ for the second drug. The time of drug administration was optimized so that the audiogenic seizure test was conducted at the time to peak effect for each experimental drug. The results were plotted as function of their ED$_{50}$ and 95% confidence intervals and isobolos were constructed in an effort to determine the nature of the observed interaction between EF1502 and tiagabine or LU32-176B (i.e., additive, antagonistic, or synergistic).

Six Hz Psychomotor Seizure Test

The ability of the EF1502 to prevent seizures induced by six Hz corneal stimulation (3 sec duration) was assessed at the time to peak effect. Current was applied by an apparatus similar to that originally described (Woodbury and Davenport, 1952). EF1502 was evaluated at a current sufficient to produce a seizure in 97% of the control animals tested (i.e., 22 mA). Prior to placement of the corneal electrodes, a drop of anesthetic/electrolyte solution (0.5% tetracaine hydrochloride in 0.9% saline) was applied to the eyes of each animal.

Six Hz seizures are characterized by a minimal clonic phase that is followed by stereotyped, automatistic behaviors described originally as being similar to the aura of human patients with partial seizures (see Barton et al., 2001 for historical review and references). Animals not displaying this behavior were considered protected.
Subcutaneous Pentylenetetrazol (s.c. PTZ)-induced Seizures and Intravenous Pentylentetrazol (i.v. PTZ) Seizure Threshold

EF1502 was tested for its ability to prevent a minimal clonic seizure induced by the chemoconvulsant PTZ administered s.c. at a dose of 85 mg/kg. Absence of a 3-sec clonic episode was used as the endpoint for protection in these three tests. PTZ was dissolved in 0.9% saline and injected in a volume of 0.01 ml/g body weight in mice. Animals were observed for at least 30 min for the presence or absence of a seizure.

Infusion of the chemoconvulsants PTZ (5 mg/ml, 0.5 ml/min) through the lateral tail vein induces clonic seizure with a loss of righting reflex in mice. The threshold for producing these seizures, calculated as mg/kg, was determined in groups of 7-12 mice 30 min after pre-treatment (i.p.) with EF1502 (4-5 doses) and the corresponding vehicle. The threshold doses for the drug treatment groups were normalised to the vehicle group and expressed as a percent increase from control. In experiments where EF1502 was co-administered with tiagabine, they were administered at the same time by i.p. injection on opposite sides of the abdomen. All data are reported as mean ± SEM and statistical analysis was performed by one-way ANOVA.

Amygdala Kindling

Rats were anaesthetised with Hypnorm (midazolam, 5 mg/ml), Dormicum (fluanisone, 10 mg/ml; fentanyl, 0.315 mg/ml), and H2O in a ratio of 1:1:2 administered s.c. in a volume of 0.2 ml/0.1kg prior to stereotaxic implantation of tri-polar electrodes (Plastics One) into the right basolateral amygdala (AP: -2.8 mm, L: -4.8, V: -8.7, measured from bregma; Paxinos and Watson, 1997). After surgery the animals received
the analgetic Rimadyl (5 mg/kg) and were subsequently allowed to recover for two weeks before the kindling protocol was initiated.

Animals were stimulated daily with monophasic square wave pulses (2 sec, 50 Hz) using an Ellegaard Systems (Denmark) stimulator. The intensity of the stimulus for kindling was determined by increasing the stimulus in 25 µA steps until an afterdischarge was observed (EEG recorded using Spike 2 software); this current afterdischarge threshold was then used for kindling. Stimulations were then delivered daily until five consecutive grade 5 seizures were observed; at this point the animals were considered to be fully kindled.

Assessment of Drug Effects on Afterdischarge Threshold

The threshold for induction of an afterdischarge was determined in kindled rats using an ascending stairstep procedure; using 10 µA as the initial stimulus, steps of 10 µA were applied at 1 min intervals until an afterdischarge was observed. Afterdischarges were defined as being EEG spikes at least twice the amplitude of the pre-stimulus recording, with a frequency greater than 1/s, for a minimum duration of 3 sec.

The effect of EF1502 on afterdischarge threshold was determined 24 h later, 30 min after administration of EF1502 (0.3 – 2.5 mg/kg, s.c.). The afterdischarge threshold was determined using 10 µA steps, with an initial stimulus 20 µA less than that previously measured for each rat. In addition to observing the afterdischarge threshold, the duration of the afterdischarge, the severity of the seizure according to the Racine scale (Racine, 1972), and the number of rats displaying fully generalized seizures (Racine scale ≥ 3) were also recorded. The ED50 and 95% confidence interval for protection.
against the fully kindled secondarily generalized seizure (Racine Scale ≥ 3 was then calculated by Probit analysis (Finney, 1971).

**Pilocarpine Seizure Test**

Groups of 8 mice were injected with pilocarpine (250 mg/kg, i.p.) 30 min after s.c. administration of EF1502 or vehicle; the dose of pilocarpine was chosen from a previously constructed dose-response curve, to induce convulsions in 97% of control animals (data not shown). The percentage of animals per group that exhibited clonic convulsions within 30 min was recorded and results were compared by Fisher’s exact probability test.

**Behavioral Impairment**

For those studies conducted at the University of Utah, minimal motor impairment was identified in mice by the rotarod procedure as described previously (Dunham and Miya, 1957). Inability of the mouse to maintain its equilibrium for one min in each of three trials on this rotating rod (6 r.p.m.) was used as an indication of such impairment. The effect of EF1502 on rotarod impairment was then quantitated in groups of 4-8 mice per dose level. Varying doses of each substance was administered to groups of mice until at least two points were established between the limits of 0 and 100% impairment. The dose of drug required to produce the desired endpoint in 50% of animals tested (i.e., the TD\textsubscript{50}) and the 95% confidence interval was then calculated by a computer program based on the method described by Finney (1971).
In combination studies conducted at H. Lundbeck A/S designed to assess whether EF1502 enhanced the behavioral toxicity of tiagabine, a modified rotarod procedure was employed. For this test, groups of 8 untrained mice were tested for 6 runs (30 sec) on a 75 mm diameter rotarod rotating at 17 r.p.m. (Rotamex 4/8, Columbus Instruments, Ohio, USA) 30 min after i.p. administration of drug or vehicle. For each mouse, the total time spent on the rotarod was recorded and mean ± SEM was calculated for each group. Significant differences between drug-treated and vehicle-treated groups were determined by one-way ANOVA.
Results

Inhibition of GABA Uptake

The inhibition profiles of tiagabine and the \textit{exo}-THPO analogs (see Figure 1) on GABA uptake in neurons and astrocytes as well as HEK cells transfected with the cloned mouse GABA transporters mGAT1-4 obtained in a recently conducted study (Clausen et al., 2005) are summarized in Table 1. It can be seen that EF1502 (the dithienyl-butenyl analog of \(N\)-methyl-\textit{exo}THPO), in contrast to tiagabine (the dithienyl-butenyl analog of nipecotic acid), exhibited pronounced inhibitory activity towards mGAT2; whereas mGAT3 and mGAT4 were only weakly inhibited. It should be noted that EF1500 as well as LU-32-176B, both of which are lipophilic analogs of \textit{exo}THPO, and not of \(N\)-methyl-\textit{exo}THPO, did not display any affinity for mGAT2-4 but only inhibited GABA transport mediated by mGAT1. All of the compounds potently inhibited GABA uptake in neurons and astrocytes. Of the drugs tested, tiagabine was approximately 10-fold more potent than the \textit{exo}THPO analogs. No neuronal or glial selectively was observed for any of the compounds tested. The kinetics of the inhibition was investigated for EF1502, an analog of \(N\)-methyl-\textit{exo}-THPO, with regard to GABA uptake in HEK cells expressing mGAT1 or mGAT2. As shown in Table 2, EF1502 was found to act as a non-competitive inhibitor of both mGAT1 and mGAT2.

Based on this information, cultured neocortical neurons preloaded with \([\textsuperscript{3}H]\)GABA were used to investigate whether GABA release could be stimulated by EF1502. The cells were superfused and the addition of a high concentration of non-radioactive GABA (i.e., 200 \(\mu\)M) to the superfusion medium led to a pronounced stimulation of release of radioactive GABA caused by homoexchange (Figure 2). When
200 µM EF1502 was used in place of GABA no increase in the release of [³H]GABA was seen (Figure 2). These results demonstrate a lack of heteroexchange between [³H]GABA and EF1502.

**Anticonvulsant Profile**

EF1502 was evaluated in a battery of anticonvulsant models to assess its ability to block both generalized and partial seizures in mice and rats (Table 3). EF1502 was effective in preventing sound-induced seizures in the Frings audiogenic seizure-susceptible mouse model of reflex epilepsy. EF1502 was found to block both tonic extensor and clonic seizure components of the audiogenic seizure. Although less potent (ED₅₀: 4.4 vs. 0.55 mg/kg, i.p., for EF1502 and tiagabine, respectively), EF1502 was equally effective to tiagabine in this reflex seizure model. As shown in Table 3, the anticonvulsant activity of EF1502 was associated with the R-isomer; i.e., the S-isomer was found to be inactive at doses up to 20 mg/kg. In a separate model, EF1502 was found to afford protection against pilocarpine-induced seizures with 100% of the animals being protected at 10 mg/kg. In the same test, tiagabine was slightly more potent offering complete protection against clonic seizures at 2.5 mg/kg.

EF1502 displayed dose-dependent protection against clonic seizures induced by s.c. PTZ and limbic seizures induced by six Hz corneal stimulation. In the s.c. PTZ test, EF1502 prevented clonic seizures (ED₅₀: 18.6 mg/kg). In addition, EF1502, at a dose as low as 2.5 mg/kg, i.p., produced a 17% increase in the i.v. PTZ threshold. In this particular test, EF1502 was equipotent to tiagabine (results not shown).
EF1502 was also found to prevent limbic seizures in two different rodent models of partial epilepsy (i.e., the six Hz psychomotor seizure test and the amygdala kindled rat model). When compared to results obtained in the Frings audiogenic seizure model, EF1502 was only slightly less potent in the six Hz psychomotor seizure test than in the Frings audiogenic seizure model (ED₅₀s: 4.4 vs. 10.4 mg/kg, i.p., respectively).

In the amygdala kindled rat model of partial epilepsy, EF1502 produced a dose-dependent reduction in both the electrographic afterdischarge duration and behavioral seizure score without affecting the afterdischarge threshold (Table 4). At the highest dose tested (2.5 mg/kg), EF1502 significantly decreased the afterdischarge duration by 50% and reduced the seizure score from 5 ± 0 to 2.6 ± 0.6.

**Combination Studies**

In an effort to assess whether the mGAT2 inhibitory properties of EF1502 contributed to its anticonvulsant activity, an isobologram study was conducted in the Frings audiogenic seizure-susceptible mouse. As shown in Figure 3A, when the two mGAT1-selective inhibitors (LU-32-176B and tiagabine) were combined, an additive anticonvulsant effect was observed (i.e., experimental results fell along the predicted line of additivity). In contrast, when the mixed mGAT1 and mGAT2 inhibitor EF1502 was combined with tiagabine or LU-32-176B, a synergistic interaction was observed (Figure 3B,C; i.e., experimental results fell to the left of the line of additivity). In a subsequent study, the combination of minimally active doses of EF1502 and tiagabine were found to produce a highly significant synergistic anticonvulsant action in the i.v. PTZ threshold test (Figure 4). Collectively, these results support the hypothesis that mGAT2 plays a
role in regulating brain excitability and that an inhibitor of mGAT2 possesses anticonvulsant activity.

**Behavioral Impairment**

The effect of EF1502 on motor performance was evaluated in two different variants of the rotarod impairment model. In the first study, the ability of a mouse to maintain balance on a 2.5 cm diameter knurled rod rotating at 6 r.p.m. was evaluated. The dose producing impairment in 50% of the mice tested (i.e., TD$_{50}$) was found to be 57 mg/kg (Table 3). In contrast, the TD$_{50}$ for tiagabine in this test was 1.3 mg/kg, i.p. In the second model, the total time spent on a 7.5 cm diameter rod rotating at 17 r.p.m. was assessed over six separate sessions each lasting 30 sec. As shown in Figure 5, doses of EF1502 as high as 40 mg/kg did not negatively impair performance in this test. In contrast, tiagabine produced marked impairment at doses of 5 mg/kg and higher (Figure 5). Interestingly, when EF1502 was tested in combination with tiagabine, the degree of impairment was not greater than that observed with tiagabine alone (Figure 5).
Discussion

In contrast to the parent compounds THPO and N-methyl-THPO, of which the latter has been shown to be a glial selective GABA uptake inhibitor (Falch et al, 1999; White et al., 2002), the lipophilic analogs of these compounds described here did not exhibit cell-type selectivity (Clausen et al., 2005). However, EF1502, the lipophilic analog of N-methyl-exo-THPO, was found to display significant affinity for mGAT2 as well as mGAT1. This was not the case for EF1500, its non-N-methylated counterpart (Clausen et al., Bioorganic Med. Chem., Submitted, June 2004). That this N-methyl group (see Figure 1) is an important determinate of GAT2 inhibitory activity is underlined by the finding that exchanging the lipophilic moiety of the molecule (i.e., EF1500 and LU-32-176B) did not by itself affect the affinity of the compound toward mGAT1 and mGAT2.

The finding that EF1502 was unable to stimulate the release of preloaded GABA in cultured neocortical neurons strongly suggests that it does not act as a substrate for the carrier. This is in line with the finding that it acted as a non-competitive inhibitor of GABA uptake in HEK cells expressing mGAT1 or mGAT2. It also agrees with the previous observation that analogous lipophilic analogs of nipecotic acid, which in itself is a substrate for the carrier (Larsson et al., 1983), do not act as substrates (Larsson et al., 1988; Braestrup et al., 1990). It is of interest to note that N-methyl-THPO was previously found to be a competitive inhibitor of mGAT1 (White et al., 2002); whereas, in the present study its lipophilic analog EF1502 was found to be a non-competitive inhibitor of mGAT1. This strongly suggests that the addition of the lipophilic side chain induces a molecular restraint that interferes with the interaction between the GABA mimetic part of the molecule and the GABA binding site of the carrier protein. Since such an alternative
binding site would not have any affinity for GABA, this kinetic analysis would indicate a mechanism of action of EF1502 that is non-competitive. Such a model would be compatible with the finding that tiagabine, having the same lipophilic side chain (Figure 1), binds to a site overlapping but not identical to the GABA recognition site (Braestrup et al., 1990).

Given the unique properties of EF1502 as an mGAT1 and mGAT2 GABA uptake inhibitor, it was of interest to assess whether it possessed any unique attributes as an anticonvulsant. Results obtained from a series of studies in well-established anticonvulsant models demonstrated that EF1502 possessed a broad spectrum anticonvulsant profile consistent with that of tiagabine. Furthermore, the results from these studies suggest that EF1502, like tiagabine, exerts its anticonvulsant action by elevating seizure threshold.

Further examination found EF1502 to be active in two models of human partial epilepsy; i.e., six Hz psychomotor seizure test and the amygdala kindled rat. The former seizure test possesses a unique pharmacological profile in that the characteristic seizure induced by low frequency stimulation is refractory to phenytoin, carbamazepine, lamotrigine, and topiramate but not to valproic acid and the novel antiepileptic drug levetiracetam (Barton et al., 2001). Another model where EF1502 showed marked anticonvulsant activity was the pilocarpine model of acute generalized seizures. Carbamazepine, lamotrigine, and phenytoin are ineffective in this model even at doses causing marked ataxic effects (Watson, 2001). To characterize EF1502’s potential for the treatment of partial epilepsy, it was evaluated in the traditional amygdala kindled rat model of partial epilepsy. In this model, it was demonstrated to decrease electrographic
seizure duration and behavioral seizure severity. These results suggest that EF1502 would be effective against primary partial seizures and partial seizures secondarily generalized. Finally, it should be noted that the anticonvulsant activity of EF1502 was restricted to its \(R\)-stereoisomer. This finding is consistent with previous results, wherein inhibition of GABA uptake was also found to be stereoselective (Clausen et al., 2005).

Interestingly, EF1502 appears to possess a tolerability profile that is superior to that of tiagabine. For example, in the rotarod performance tests conducted, doses substantially exceeding anticonvulsant effective doses did not appear to produce any notable impairment. In contrast, at a dose that was only slightly higher than its anticonvulsant dose in the i.v. PTZ threshold test, tiagabine produced a significant dose-dependent impairment in the rotarod test (Figure 5). Thus, based on these initial findings it would appear that EF1502 offers some beneficial improvement over the GAT1-selective inhibitor tiagabine.

At the present time, the role of mGAT2 within the brain is not understood. This transporter is thought to regulate the uptake of the osmo-regulator betaine (see Borden et al., 1995 for review and references). In an effort to determine whether inhibition of mGAT2 might contribute the anticonvulsant action of EF1502, three separate combination studies with tiagabine were undertaken. The first of these, conducted in the Frings audiogenic seizure-susceptible mouse, clearly demonstrated that the combination of the GAT1 and GAT2 inhibitor EF1502 with the GAT1-selective inhibitors tiagabine or LU-32-176B results in a synergistic anticonvulsant action (Figure 3B,C). This conclusion, is supported by the negative finding wherein the combination of two GAT1-selective inhibitors (i.e., tiagabine and LU-32-176) in the same model resulted in only an
additive effect. That this synergism is indeed related to the difference in the affinity for mGAT1 and mGAT2 and not to differences in chemical structures of the GABA-mimetic moiety is supported by the fact that the GAT1 inhibitors tiagabine and LU-32-176B, which are structurally different from each other, both with regard to their lipophilic side chain and their GABA mimetic moiety (Figure 1), did not exhibit synergism. This is further substantiated by the finding that synergism could be demonstrated with EF1502 in combination with either of the structurally different mGAT1 inhibitors tiagabine and LU-32-176B. Additional support for this conclusion is provided by the combination study conducted in the i.v. PTZ seizure threshold test. In this study, minimally effective doses of EF1502 and tiagabine, when administered together, resulted in a greater than additive effect. Collectively, these results suggest that inhibition of mGAT2 by EF1502 contributes significantly to its overall anticonvulsant activity. It is worth noting that the synergy between EF1502 and tiagabine or LU-32-176B could be related to pharmacokinetic interaction between these compounds. Such an interaction could theoretically lead to an increase in the intracerebral concentration and an apparent supra-additive effect. However, the fact that synergism was observed after combination of EF1502 with either of the two mGAT1 inhibitors, but was not seen with the combination of tiagabine and LU-32-176B, the structures of which are totally different (Figure 1), makes this highly unlikely. In addition, rotarod impairment was not increased when EF1502 was combined with tiagabine. Such an effect would have been predicted by a purely pharmacokinetic interaction.

Based on Northern blot studies in human brain and in situ hybridization studies in mouse brain, the betaine transporter (i.e., mGAT2) appears to be primarily localized to
the extrasynaptic component (Borden et al., 1995). However, ultrastructural immunocytochemical studies have yet to add more details to these findings. On the other hand, when microinjected into cultured hippocampal neurons, mGAT2 was reported to co-localize with MAP2 but not with synapsin (Ahn et al., 1996). Moreover, after transfection of mGAT2 into Madin Darby canine kidney cells, mGAT2 is sorted to the the basolateral surface of the cells (Ahn et al., 1996). These findings support a somatodendritic localization of mGAT2 and it seems reasonable to hypothesize that mGAT2 may play a role in regulating extrasynaptic GABA levels. Thus, inhibiting mGAT2 would be expected to increase extrasynaptic GABA that could then activate nonsynaptic GABA_A and GABA_B receptors.

Interestingly, EF1502 was found to possess an anticonvulsant profile similar to that of the non-selective GAT2-4 inhibitors NNC 05-2045 and NNC 05-2090 (Dalby et al., 1997). In this study, the anticonvulsant activity of these compounds was attributed to inhibition of mGAT3 and mGAT4 in spite of the fact that these two inhibitors were somewhat more potent inhibitors of mGAT2 (Thomsen et al., 1997). Hence, given that EF1502 lacks affinity for mGAT3 and mGAT4, an alternative conclusion would be that inhibition of mGAT2 did indeed contribute to the anticonvulsant efficacy of these two inhibitors of GABA transport. Clearly, the development of a purely selective, peripherally active, mGAT2 inhibitor could resolve this issue.

It is of particular interest that synergy was obtained in the anticonvulsant studies but not in the rotarod impairment study conducted. Lack of a synergistic effect in this study might suggest that a selective mGAT2 inhibitor would offer some distinct advantage over a non-selective mGAT1/mGAT2 inhibitor when it comes to tolerability.
However, this hypothesis will only be substantiated or refuted at the time that a peripherally active mGAT2-selective inhibitor becomes available for testing. Overall, the results of the present investigation suggest that EF1502 possesses a favorable anticonvulsant and tolerability profile and support the continued development of EF1502 and its analogs.
Acknowledgements

The authors gratefully acknowledge the expert technical assistance of Lone Rosenquist, Kirsten Thuesen, and Laura Webb and the editorial assistance of Irene Kamerath. We further acknowledge Nathan Nelson (Tel Aviv University, Israel) for providing the mGAT1 and mGAT2 clones used in this study.
References


of 4-N-Methylamino-4,5,6,7-tetrahydrobenzo[d]isoxazol-3-ol Analogues. Bioorganic & Medicinal Chemistry In Press


Footnotes

Footnote to Title

This study was supported by the Danish State Medical Research Council (20-00-1011), the Lundbeck Foundation, and the National Institute of Neurological Disorders and Stroke (NO1-NS-9-2313).

Numbered footnotes

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Figure Legends

Figure 1: Chemical structures of nipecotic acid, exo-THPO, N-methyl-exo-THPO and their lipophilic derivatives tiagabine, LU-32-176B, EF1500, and EF1502.

Figure 2: Cerebral cortical neurons were cultured for 7 days and preloaded with \(^{3}\text{H}\)GABA as described in Experimental Procedures. Subsequently the culture dishes were placed in the superfusion system (see Experimental Procedures) and fractions were collected and the radioactivity determined. As indicated by the shaded bars, the cells were exposed to 200 µM GABA (fractions 7-10) or 200 µM EF-1502 (fractions 23-26) in order to assess a possible exchange with the intracellular pool of radioactively labelled GABA. Results are expressed as the average of four independent superfusion experiments ± SEM values.

Figure 3: Additive anticonvulsant effect of LU-32-176B when combined with tiagabine (3A) and synergistic anticonvulsant effect of EF1502 when combined with tiagabine (3B) or LU-32-176B (3C) in the Frings audiogenic seizure-susceptible mouse. For these studies, varying doses (administered as a fraction of their predetermined ED\(_{50}\) value) of either LU-32-176B, tiagabine, or EF1502 were administered i.p. The effect of any given dose-level on the anticonvulsant efficacy of the secondary drug was established by re-determining the ED\(_{50}\). The time of each drug administration was optimized in order that all anticonvulsant testing was conducted at the time to peak effect for each drug. The results are plotted as a function of their ED\(_{50}\) and 95% confidence intervals.
predicted ‘line of additivity’ and 95% confidence interval connects the individual ED50 values for each drug tested independently.

**Figure 4:** Synergistic anticonvulsant effect of EF1502 and tiagabine in the i.v. PTZ seizure threshold test. The effect of EF1502 (1.38 mg/kg) and tiagabine (0.63 and 1.38 mg/kg) on i.v. PTZ seizure threshold was determined 30 min after i.p. administration in three separate groups of mice (n = 7-12 mice/group). The combined effect of EF1502 and tiagabine on i.v. PTZ seizure threshold was then assessed. At the doses tested, EF1502 and tiagabine did not increase i.v. PTZ threshold when administered alone; however, combination of EF1502 and tiagabine resulted in a significant elevation of seizure threshold above that which would have been predicted by a purely additive interaction. Results were normalized to the vehicle-treated group and are expressed as the mean ± the SEM percent increase in seizure threshold. Statistical significance was determined by one-way ANOVA.

**Figure 5:** Lack of negative interaction between EF1502 and tiagabine in the rotarod performance test. Thirty min after vehicle or drug administration, groups of untrained mice (n = 8 mice/group) were tested in six separate trials each lasting for 30 sec (180 sec total). For this study, individual mice were placed on a 75 mm diameter rotarod rotating at 17 r.p.m. The cumulative time (sec) ± SEM that the mouse spent on the rod during the six individual trials was then averaged and plotted as a function of the dose of EF1502 (filled squares) or tiagabine (filled triangles) administered. For the combination study, separate groups of mice received 15 mg/kg EF1502 and a single dose of tiagabine (1.3, 2.5, 10, and 20 mg/kg; inverted triangle). Both drugs were administered s.c. Thirty min
after drug administration, their performance on the rotarod was assessed and the results plotted as the mean time (sec) ± SEM spent on the rotating rod.
Table 1: IC₅₀ values for inhibition of GABA uptake in neurons, astrocytes and cloned mouse GABA transporters expressed in HEK cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Neurons</th>
<th>Astrocytes</th>
<th>mGAT1</th>
<th>mGAT2</th>
<th>mGAT3</th>
<th>mGAT4</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF1502</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>22</td>
<td>&gt;300</td>
<td>&gt;300</td>
</tr>
<tr>
<td>EF1500</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>130</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>LU-32-176B</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>&gt;100</td>
<td>300</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Tiagabine</td>
<td>0.36</td>
<td>0.18</td>
<td>0.8</td>
<td>300</td>
<td>&gt;300</td>
<td>800</td>
</tr>
</tbody>
</table>

Values are from Clausen et al., J. Med. Chem., Submitted, March 2004
Table 2: Kinetic characterization of GABA transport in HEK cells expressing mGAT1 or mGAT2 by EF1502.

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol x min$^{-1}$ x mg$^{-1}$)</th>
<th>$K_i$ (µM)</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12 ± 4</td>
<td>1.65 ± 0.19</td>
<td>4</td>
<td>Non-competitive</td>
</tr>
<tr>
<td>EF1502</td>
<td>17 ± 5</td>
<td>0.73 ± 0.11*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>55 ± 6</td>
<td>0.41 ± 0.04</td>
<td>5</td>
<td>Non-competitive</td>
</tr>
<tr>
<td>EF1502</td>
<td>52 ± 9</td>
<td>0.21 ± 0.02*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HEK cells transiently expressing mGAT1 and mGAT2 were prepared as detailed in methods and the kinetic analysis of GABA transport performed in the absence or presence of 5 µM EF1502 using a series of GABA concentrations as described in Methods. Statistically significant differences from the control (Students t-test) are indicated by asterisks (* p <0.01).
TABLE 3. Anticonvulsant Profile of EF1502 Following Systemic Administration

<table>
<thead>
<tr>
<th>Strain/Species</th>
<th>Test</th>
<th>Time of test, Route</th>
<th>ED$<em>{50}$ or TD$</em>{50}$ (mg/kg)</th>
<th>95% Confidence Interval</th>
<th>P.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF#1 mouse</td>
<td>Rotarod</td>
<td>30’, i.p.</td>
<td>57.2</td>
<td>37.9 - 91.4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>scPTZ</td>
<td>30’, i.p.</td>
<td>18.6</td>
<td>12.4 - 27.1</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>6 Hz</td>
<td>30’, i.p.</td>
<td>10.4</td>
<td>6.8 - 15.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Frings mouse</td>
<td>Rotarod</td>
<td>30’, i.p.</td>
<td>18.2</td>
<td>14.7 - 22.9</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>AGS</td>
<td>30’, i.p.</td>
<td>4.4 ($R,S$)</td>
<td>3.2 - 5.5</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>AGS</td>
<td>30’, i.p.</td>
<td>3.1 ($R$)</td>
<td>2.0 - 3.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AGS</td>
<td>30’, i.p.</td>
<td>&gt;20 ($S$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMRI mouse</td>
<td>Pilocarpine</td>
<td>30’, s.c.</td>
<td>100% protection at 10 mg/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wistar rat</td>
<td>Amygdala</td>
<td>30’, s.c.</td>
<td>0.8</td>
<td>0.07 - 4.9</td>
<td>&gt;4</td>
</tr>
</tbody>
</table>

For each of the anticonvulsant studies conducted, the median effective (ED$_{50}$) or median toxic (TD$_{50}$) was determined at the time to peak effect according to the methods described in the text.

The pilocarpine and amygdala kindled rat studies were conducted at H. Lundbeck A/S. All other anticonvulsant studies were conducted at the University of Utah. C.I. - Confidence Interval  
P.I. - Protective Index (TD50/ED50)
TABLE 4: Effect of EF1502 on Amygdala-Kindled Rat

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>ADT (µA)</th>
<th>ADD (sec)</th>
<th>BSS (Racine Scale 0 - 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>78.3 ± 25.2</td>
<td>81 ± 9.5</td>
<td>5 ± 0</td>
</tr>
<tr>
<td>0.30</td>
<td>55.7 ± 11.3</td>
<td>101 ± 15.6</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td>0.63</td>
<td>66.9 ± 10.1</td>
<td>54.8 ± 18.5</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>2.5</td>
<td>112 ± 20.1</td>
<td>40.5 ± 15.6</td>
<td>2.6 ± 0.6 *</td>
</tr>
</tbody>
</table>

OneWay ANOVA: F(3,28)=2.2; P >0.1  F(3,28) = 3.02; P <0.05  H =10.9 (3 df) P = 0.01

The effect of EF1502 on afterdischarge duration (ADD), afterdischarge duration (ADD), and behavioral seizure score (BSS) was determined 30 min after subcutaneous administration of varying doses of EF1502 according to the procedures described in the methods. Parametric one-way ANOVA was used to assess effect of treatment for ADT and ADD, whilst one Way ANOVA on ranks was used for the seizure score. Asterisk indicates P <0.05 compared to vehicle using Dunn’s method for multiple comparisons versus vehicle.
Figure 1

Nipecotic acid

Exo-THPO

N-methyl-exoTHPO

Tiagabine

Lu-32-176B

EF1500

EF1502
Figure 2

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 3

A

![Graph showing Tiagabine (mg/kg, i.p.) vs. LU-32-176B (mg/kg, i.p.)](image)

B

![Graph showing Tiagabine (mg/kg, i.p.) vs. EF1502 (mg/kg, i.p.)](image)

C

![Graph showing Ef1502 (mg/kg, i.p.) vs. LU-32-176B (mg/kg, i.p.)](image)
Figure 4

![Graph showing i.v. PTZ threshold (% control) for different treatments: Control, EF1502, TGB (0.63), TGB (1.38), EF + 0.63 TGB, EF + 1.38 TGB.]

- P < 0.001 for Control vs. EF1502
- P < 0.001 for Control vs. TGB (0.63)
- P < 0.001 for Control vs. TGB (1.38)
- P < 0.01 for Control vs. EF + 0.63 TGB
- P < 0.01 for Control vs. EF + 1.38 TGB
Figure 5

[Graph showing the relationship between Dose (mg/kg) and Rotarod Performance (sec on rod)].