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Correlation between Anticonvulsant Activity and Inhibitory Action on Glial γ-Aminobutyric Acid Uptake of the Highly Selective Mouse γ-Aminobutyric Acid Transporter 1 Inhibitor 3-Hydroxy-4-amino-4,5,6,7-tetrahydro-1,2-benzisoxazole and Its *N*-Alkylated Analogs

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ABSTRACT

The inhibitory effect of 3-hydroxy-4-amino-4,5,6,7-tetrahydro-1,2-benzisoxazole (*exo*-THPO) and its *N*-methylated (*N*-methyl-*exo*-THPO) and *N*-ethylated (*N*-ethyl-*exo*-THPO) analogs, derived from γ-aminobutyric acid (GABA) and 4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol (THPO) on GABA transport was investigated using cultured neocortical neurons (GABA-ergic) and astrocytes and cloned mouse GABA transporters GAT1–4 expressed in human embryonic kidney (HEK) 293 cells. Anticonvulsant activity was assessed after i.c.v. administration to Frings audiogenic seizure-susceptible mice. Anticonvulsant activity of the *O*-pivaloyloxymethyl prodrug of *N*-methyl-*exo*-THPO was assessed after i.p. administration. Results from these studies were compared with those obtained from similar studies with the novel anticonvulsant drug tiagabine, which acts via inhibition of GABA transport. *exo*-THPO and its *N*-alkyl analogs inhibited neuronal, astrocytic, and GAT1-mediated GABA transport but not GABA uptake mediated by GAT2–4. *N*-Methyl-*exo*-THPO was 8-fold more potent as an inhibitor of astrocytic versus neuronal GABA uptake. The IC_{50} value for inhibition of GABA uptake by GAT1 closely reflected its IC_{50} value for inhibition of neuronal uptake. Tiagabine was approximately 1000-fold more potent than *exo*-THPO and its alkyl derivatives as an inhibitor of GABA uptake in cultured neural cells and GAT1-expressing HEK 293 cells. *exo*-THPO, its alkylated analogs, and tiagabine displayed a time- and dose-dependent inhibition of audiogenic seizures after i.c.v. administration. *N*-Methyl-*exo*-THPO was the most potent anticonvulsant among the *exo*-THPO compounds tested and only slightly less potent than tiagabine. The findings suggest a correlation between anticonvulsant efficacy and selective inhibition of astroglial GABA uptake. Furthermore, results obtained with the *N*-methyl-*exo*-THPO prodrug demonstrate the feasibility of developing a glial-selective GABA uptake inhibitor with systemic bioavailability.

Within the central nervous system (CNS), γ-aminobutyric acid (GABA) serves as the principal inhibitory neurotransmitter. Once released from the presynaptic terminal, GABA binds to both presynaptic and postsynaptic GABA receptors, which are coupled to either a G protein-associated K^+ channel ($GABA_B$ receptors) or form Cl^--permeable ion channels ($GABA_A$ receptors). Alterations in GABA-ergic function have been implicated in a number of CNS disorders, including epilepsy, migraine, bipolar disorder, anxiety, and depression. As such, it is not surprising that the $GABA_A$ receptor has been the target for numerous therapeutic entities, including the benzodiazepines, barbiturates, and neurosteroids. Collec-

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ABBREVIATIONS: CNS, central nervous system; GABA, γ-aminobutyric acid; *exo*-THPO, 3-hydroxy-4-amino-4,5,6,7-tetrahydro-1,2-benzisoxazole; AGS, audiogenic seizures; dBcAMP, dibutyryl-cyclic AMP; HEK, human embryonic kidney; GAT, γ-aminobutyric acid transporter; *N*-methyl-*exo*-THPO, 3-hydroxy-4-(methylamino)-4,5,6,7-tetrahydro-1,2-benzisoxazole; *N*-ethyl-*exo*-THPO, 3-hydroxy-4-(ethylamino)-4,5,6,7-tetrahydro-1,2-benzisoxazole; BBB, blood-brain barrier; THPO, 4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol.

tively, these findings demonstrate the critical role played by GABA within the CNS and underscore the therapeutic impact of manipulating GABA-ergic neurotransmission within the CNS (for review and references, see Treiman, 2001).

Allosteric $GABA_A$ receptor modulators such as the benzodiazepines and barbiturates have clearly played an important role in the treatment of epilepsy. However, the long-term utility of the benzodiazepines as anticonvulsants is limited by the rapid development of tolerance (for review and references, see Schmidt, 1995). Today, the primary use of the benzodiazepines as anticonvulsants is in the acute treatment of status epilepticus. Although effective as broad-spectrum antiseizure drugs, the clinical utility of the barbiturates is limited by the development of cognitive impairment and behavioral modifications [for reviews, see Devinsky (1995) and Mattson (1995)].

The action of neuronally released GABA is terminated by rapid uptake of GABA into surrounding neurons and astrocytes by selective GABA transporters (Schousboe and Kanner, 2002). Molecular cloning has identified four distinct GABA transporters (GAT1–4) that display regionally distinct distribution within the CNS (Gadea and Lopez-Colome, 2001). Attempts to design and synthesize specific inhibitors of GABA transport led to the successful identification of several investigational agents with demonstrated anticonvulsant activity in animal seizure models (Yunger et al., 1984; Schousboe et al., 1986; Krogsgaard-Larsen et al., 1987; White et al., 1993; Borden et al., 1994; Suzdak and Jansen, 1995; Bolvig et al., 1999). The GAT1-selective GABA uptake inhibitor tiagabine was subsequently demonstrated to possess clinical efficacy for the treatment of partial epilepsy in randomized clinical trials (Suzdak and Jansen, 1995) and was approved for the adjunctive treatment of partial epilepsy in 1997. The approval and subsequent registration of tiagabine as an antiepileptic drug clearly demonstrate the therapeutic potential associated with GABA uptake inhibitors. Tiagabine is an effective and safe drug for the management of epilepsy; however, it possesses a less than desirable pharmacokinetic profile. For example, it displays a short half-life (i.e., 7–9 h), which can be reduced even further (i.e., 2–3 h) by enzyme-inducing antiepileptic drugs, thereby necessitating three-time-a-day dosing (Genton et al., 2001). Tolerability to tiagabine seems to be limited by the presence of mild-to-moderate side effects, including dizziness, fatigue, and confusion (Genton et al., 2001). As such, the clinical utility of tiagabine is somewhat limited and the development of a better tolerated GABA uptake inhibitor with a more favorable pharmacokinetic profile could provide some distinct advantage over tiagabine.

It has been proposed that because GABA-mediated neurotransmission to a considerable extent relies on the recycling of GABA by reuptake into the presynaptic nerve ending it may be advantageous to selectively inhibit astrocytic GABA uptake to enhance GABA-ergic activity (Schousboe et al., 1983; Schousboe, 2000). This notion seems to be experimentally supported by the findings that selective inhibitors of neuronal GABA uptake are proconvulsants, whereas even marginally selective glia inhibitors are anticonvulsants and are able to increase the levels of extrasynaptic GABA (Schousboe et al., 1983; Gonsalves et al., 1989a,b; Juhasz et al., 1997). By selectively inhibiting astrocytic GABA uptake, presynaptic GABA levels are increased without impairing GABA metabolism. In contrast, selective inhibition of neuronal GABA transport leads to a disruption of GABA metabolism and ultimately a reduction in the total pool of releasable GABA (for review and references, see Schousboe et al., 1983).

The present study was undertaken to further investigate the cell type selectivity of analogs of 3-hydroxy-4-amino-4,5,6,7-tetrahydro-1,2-benzisoxazole (*exo*-THPO) that were previously reported to exhibit some cell type selectivity as GABA uptake inhibitors (Falch et al., 1999). Moreover, studies were conducted to characterize their anticonvulsant potential in the audiogenic seizure-susceptible (AGS) Frings mouse and to correlate their anticonvulsant activity with their potency as GABA transport inhibitors. Results obtained with *exo*-THPO and its two *N*-alkyl analogs were compared with results obtained with the clinically effective antiseizure drug and prototype GAT1 inhibitor tiagabine.

Experimental Procedures

Materials

Newborn mice and 15-day-old mouse embryos were obtained from Bomholtgaard (Ry, Denmark). Plastic tissue culture dishes were purchased from NUNC A/S (Roskilde, Denmark) and fetal calf serum from Sera-Lab (Sussex, UK). Poly-D-lysine (molecular weight >300,000), trypsin, soybean trypsin inhibitor, dibutyryl cyclic AMP (dBcAMP), DNase, cytosine arabinoside, and amino acids were obtained from Sigma-Aldrich (St. Louis, MO); insulin from Novo Nordisk (Bagsværd, Denmark), and penicillin from Leo (Ballerup, Denmark). [^{3}H]GABA (79.0 Ci mmol^{-1}) was from DuPont (Frankfurt, Germany). *exo*-THPO and its *N*-alkyl analogs were synthesized as described previously (Falch et al., 1999). Tiagabine was generously provided by Novo Nordisk. All other chemicals were of the purest grade available from regular commercial sources. The accession numbers for mouse GAT1–4 are M92378, M97632, L04663, and L04662, respectively.

Primary Cell Culture

Astrocytes were cultured essentially as described by Hertz et al. (1989b). Prefrontal cortex was taken from newborn mice and passed through Nitex nylon sieves (80-μm pore size) into a slightly modified Dulbecco's minimum essential medium as defined by Hertz et al. (1982) containing 20% (v/v) fetal calf serum and subsequently plated onto NUNC 24-well multidishes. After 14 days in culture, and during the following weeks, the serum concentration in the culture medium was reduced to 10% (v/v) and 0.25 mM dBcAMP was added. This addition of dBcAMP led to the formation of well differentiated astrocytes (Hertz et al., 1982).

Cerebral cortical neurons were isolated and cultured from 15-day-old mouse embryos. After dissociation of the tissue by trypsinization and trituration in a DNase solution containing soybean trypsin inhibitor as described by Hertz et al. (1989a), the cells were plated onto NUNC 24-well multidishes. 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 to 8 days at which time the neurons had become functionally differentiated (Hertz and Schousboe, 1987).

Transient Expression of GABA Transporters

The cDNAs encoding the four murine GABA transporters (Liu et al., 1992; Lopez-Corcuera et al., 1992) were subcloned into the mammalian expression vector pCis as detailed previously (Bolvig et al., 1999) and used for transient transfections into HEK 293 cells. HEK 293 cells were maintained in complete growth medium (minimal essential medium with Earle's salts, supplemented with 5% fetal calf serum, 1% anti-pleuro-pneumonia-like organisms, and 1% Glutamax

I, pH 7.3 under 5% CO_2). GATpCis transfections were carried out as described by Chen and Okayama (1987) and Pritchett et al. (1988).

Construction of Expression Vectors for Stable Transfection of GATs

A modified version of the bicistronic expression vector pIRES (CLONTECH, Palo Alto, CA) was constructed, pIRES-BLAS-AN. A DNA fragment containing the gene from *Aspergillus terreus* coding for the enzyme blasticidin S deaminase was inserted into the multiple cloning site B of pIRES vector, allowing the use of blasticidin as the selection marker. The multiple cloning site A of pIRES-BLAS was expanded by digestion with *Nhe*I followed by treatment with Klenow enzyme to give blunt ends. Then the complementary primers 5′-ACCGGTGATATCTCTAGAGGCGCGCCGCTAGC-3′ and 5′-GCTAGCGGCGCGCCTCTAGAGATATCACCGGT-3′ were annealed and ligated into this position of pIRES-BLAS, generating pIRES-BLAS-AN and pIRES-BLAS-NA, which have opposite orientations of the added unique enzyme restriction sites. The new multiple cloning sites AN and NA were verified by automated DNA sequencing (MWG Biotech, Ebersberg, Germany).

To stably express the four mGATs, the corresponding cDNAs were subcloned into pIRES-BLAS-AN. GAT1 cDNA was subcloned from GAT1pBSK by digestion with *Bam*HI, (blunt-ended with Klenow enzyme) and *Nhe*I and ligated into the *Eco*RV (5′) and *Xba*I (3′) sites of pIRES-BLAS-AN. GAT2 cDNA was subcloned from GAT2pBSK by digestion with *Xba*I and *Nhe*I and ligation into the *Xba*I site of pIRES-BLAS-AN. GAT3 cDNA was subcloned from GAT3pGEM4Z by digestion with *Dra*III (blunt-ended with Klenow enzyme) and *Eco*RI. The GAT3 insert was ligated into the (5′) *Eco*RI and (3′) *Mlu*I (blunt-ended with Klenow enzyme) sites of pIRES-BLAS-AN. GAT4 cDNA was subcloned from GAT4pBSK by digestion with *Xba*I and ligating the insert into the *Xba*I site of pIRES-BLAS-AN. All clones were identified by restriction enzyme analysis and subsequently constructs were confirmed by automated DNA sequencing (MGW Biotech).

Isolation of Stably Transfected Cells

Each of the four GATpIRES-BLAS-AN cDNAs was transfected into HEK 293 cells as described above for transient transfections. Cells were selected in the presence of 20 μg/ml blasticidin S (CAYLA, Toulouse, France) in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Colonies of stably transformed cells were obtained after 7 to 14 days of exposure to blasticidin S and were subsequently pooled and maintained in 2 μg/ml blasticidin S.

[^{3}H]GABA Uptake

The uptake of [^{3}H]GABA in cultured astrocytes and neurons as well as in recombinant cell systems was investigated essentially as described previously (Schousboe et al., 1977; Larsson et al., 1986a). The incubations were performed at 37°C in phosphate-buffered saline with an incubation time of 3 min. Incubations were initiated by exchanging the culture medium with incubation medium leaving the cells attached to the bottom of the wells during the entire procedure. Incubations were terminated by rapid wash with nonradioactive incubation medium. In the kinetic experiments, the GABA concentration varied over the range 1 to 1000 μM, whereas in IC_{50} studies it was constant at 1 μM. The concentrations of uptake inhibitors present during incubations in the kinetic assays are stated in Tables 2 and 3. After incubation, the cells were dissolved in 0.4 M KOH, and radioactivity (Schousboe et al., 1977) and protein concentration (Lowry et al., 1951) were determined. Protein contents were related to bovine serum albumin used as the standard.

Data and Statistical Analysis

The IC_{50} values were determined by use of SigmaPlot for Windows, version 3.02. The percentage of GABA uptake as a function of the inhibitor concentration in probit-log scale was plotted. The kinetic parameters V_{max} and K_m of the carrier-mediated high-affinity glial, neuronal, and GAT1 uptake were calculated (Larsson et al., 1986a,b) by means of a computer program for unconstrained minimization (Stewart, 1967; Dixon, 1972) or via the computer program of SigmaPlot for Windows, version 3.02, fitting to the following equation: $V = [(V_{max}S)/(K_m + S)] + kS$. In neurons, astroglial cells, and HEK 293 cells expressing the GAT subtype, the nonsaturable influx component (kS) varied somewhat between batches but was in the range of 10^{-3} to 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 simple competitive inhibition, the K_i values were calculated from the determined K_m values of the control and test situation using the following equation: $K_i = I/[(K'_m/K_m - 1]$; where I is the inhibitor concentration, K_m is the control value, and K'_m is the value determined in the presence of inhibitor.

Anticonvulsant Testing

Animals. Adult male and female Frings AGS mice (20–25 g of body weight) were obtained from an in-house breeding colony at the University of Utah (Salt Lake City, UT). Mice were housed in Association for the Assessment and Accreditation of Laboratory Animal Care-approved facilities under a constant 12-h light/dark cycle with a constant temperature of 21–23°C and a relative humidity of 30 to 50%. All animals were permitted free access to standard laboratory chow (Prolab RMH 3000; PMI Nutrition International, LLC, Brentwood, MO) and water except when removed from their home cages for testing. All mice were handled in a manner consistent with the recommendations in the National Research Council Publication Guide for the Care and Use of Laboratory Animals. All test compounds were dissolved in water, and the solutions made alkaline (pH 8–10) with 1 M NaOH. Once in solution, the final pH was adjusted to a value between 7 and 8 with 1 M HCl.

Audiogenic Seizures. The ability of *exo*-THPO and its corresponding *N*-methyl-*exo*-THPO and *N*-ethyl-*exo*-THPO analogs to prevent sound-induced seizures in the AGS Frings mouse model was assessed after i.c.v. administration (White et al., 1992). A similar study was conducted with the prototype GABA uptake inhibitor tiagabine. Each test substance was injected i.c.v. into the lateral ventricle of conscious mice with a 10-μl Hamilton syringe. Each compound was administered i.c.v. in a constant volume of 5 μl over a 10-s period. The i.c.v. route of administration was used due to the apparent poor penetration of the blood-brain barrier (BBB) by the bicyclic isoxazoles, and although tiagabine is able to cross the BBB, it was also administered i.c.v. so that a direct comparison of the results could be made.

For each of the analogs examined, time-response curves were constructed using a dose that produced a submaximal anticonvulsant effect in a group of a minimum of four mice per time point tested (5, 15, 30, 60, and 120 min). Dose-response studies were then conducted at the previously determined time of peak effect of each test substance. For these studies, individual mice were placed into a Plexiglas cylinder (diameter 15 cm; height 18 cm) fitted with an audiotransducer (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 s. 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 four to eight mice per dose level. Various 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. Minimal motor impairment was assessed by the rotarod test (Orlof et al., 1949). For this study, groups of six to eight mice per dose were tested for their ability to maintain balance on a rotating rod (6 rpm). Animals failing to remain on the rotating rod in three trials over a 60-s test period were considered toxic. The dose levels were varied until at least two points were established between the limits of 0 and 100% toxicity. The dose of drug required to produce the desired endpoint in 50% of animals tested [i.e., the ED_{50} or median toxic dose (TD_{50})] and the 95% confidence interval were then calculated by a computer program based on the method described by Finney (1971).

In addition to assessing the percentage of protection after 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 s; 2, wild running for >10 s; 3, all-limb clonus; 4, forelimb extension; and 5, forelimb and hindlimb extension.

Similar time- and dose-response studies were conducted after i.p. administration of the prodrug of *N*-methyl-*exo*-THPO (i.e., pivaloyloxymethyl derivative of *N*-methyl-*exo*-THPO).

Results

Inhibition of GABA Uptake. Previous studies on the inhibitory action of *exo*-THPO, *N*-methyl-*exo*-THPO, and *N*-ethyl-*exo*-THPO (Fig. 1) on neuronal and astrocytic GABA transport were extended to include the cloned mouse GABA transporters GAT1–4. Concentration-response curves for inhibition of GABA uptake into neurons, astrocytes, and HEK 293 cells transfected with mGAT1 were used to calculate the IC_{50} values for *exo*-THPO, its two alkylated analogs, and tiagabine. The results from this analysis are summarized in Table 1 along with inhibition data for all four compounds in GAT2–4-transfected HEK 293 cells. It can be seen that the IC_{50} values for *exo*-THPO and *N*-methyl-*exo*-THPO on mGAT1 reflect the IC_{50} values found for inhibition of GABA uptake into neurons by the two compounds. Moreover, none of the compounds inhibited GABA transport in HEK 293 cells transfected with GAT2–4. Furthermore, kinetic analysis of the inhibitory action of *exo*-THPO and *N*-methyl-*exo*-THPO on GAT1 (Table 2) demonstrated that both of these GABA analogs are competitive inhibitors of GABA uptake. Similarly, *exo*-THPO was found to display competitive inhibition kinetics for GABA uptake into neurons and astrocytes (Table 3). The results obtained with *exo*-THPO, *N*-methyl-*exo*-THPO, and *N*-ethyl-THPO were compared with the nipecotic acid derivative tiagabine (Fig. 1), which is currently in clinical use for the management of partial seizures. As can be seen from Table 1, tiagabine inhibited GABA uptake into neurons, astrocytes, and GAT1-transfected HEK 293 cells much more potently than the inhibitors derived from 4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol (THPO). Tiagabine, like *exo*-THPO and the two *exo*-THPO analogs, weakly or insignificantly inhibited GABA uptake into GAT2–4-transfected HEK 293 cells.

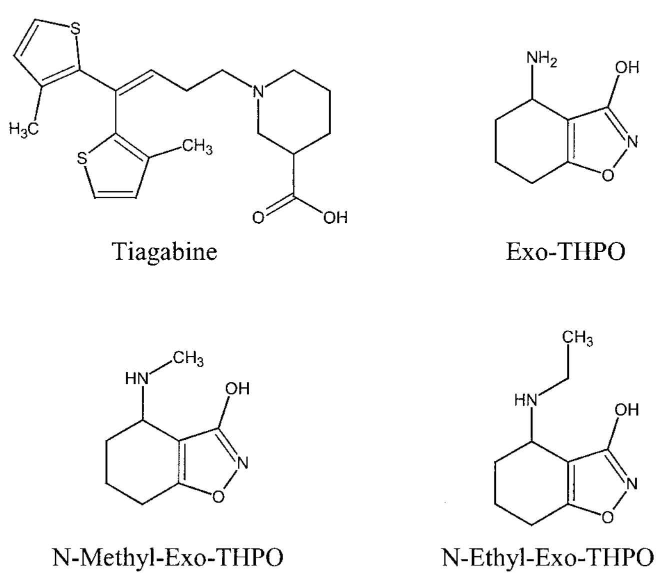

Fig. 1. Chemical structures of THPO, *exo*-THPO, and its *N*-alkyl analogs *N*-methyl-*exo*-THPO and *N*-ethyl-*exo*-THPO; the nipecotic acid analog tiagabine; and the *O*-pivaloyloxymethyl prodrug of *N*-methyl-*exo*-THPO.

Inhibition of Audiogenic Seizures. Inhibition of GABA uptake by i.c.v. administration of the GAT1-selective GABA uptake inhibitors resulted in a time- and dose-dependent inhibition of audiogenic seizures in the Frings mouse. As shown in Fig. 2, peak anticonvulsant activity for all three *exo*-THPO analogs was observed within 15 to 30 min of i.c.v. administration. In contrast, the onset of action of the prototype anticonvulsant tiagabine was delayed relative to the three *exo*-THPO analogs tested. Hence, the peak anticonvulsant activity of tiagabine was observed 2 h after an i.c.v. dose of 48.5 nmol (Fig. 2).

Slight differences in the duration of anticonvulsant activity between the three *exo*-THPO compounds and tiagabine were observed. For example, the duration of *exo*-THPO was rather brief compared with those of *N*-methyl-*exo*-THPO, *N*-ethyl-*exo*-THPO, or tiagabine. In contrast, marked anticonvulsant efficacy was still evident 2 h after i.c.v. administration of *N*-methyl-*exo*-THPO. The duration of action of *N*-ethyl-*exo*-THPO was intermediate to that of *exo*-THPO and *N*-methyl-*exo*-THPO (Fig. 2). The anticonvulsant activity of tiagabine was markedly reduced by 4 h after i.c.v. administration.

The anticonvulsant actions of tiagabine and the three compounds derived from THPO were quantitated at their respective time to peak effect. As shown in Fig. 3, all four compounds displayed a dose-dependent inhibition of audiogenic seizures and a corresponding reduction in seizure severity (data not shown). The *N*-methyl-*exo*-THPO analog was the most potent of the three members of the *exo*-THPO family tested. As summarized in Table 4, it was found to be at least 2-fold more potent than either *exo*-THPO or *N*-ethyl-*exo*-THPO. *N*-ethyl-*exo*-THPO was unique in that the degree of protection against audiogenic seizures afforded by this compound was found to plateau at a maximum protection of 75% between doses of 225 and 475 nmol i.c.v. (Fig. 3). *exo*-THPO and *N*-ethyl-*exo*-THPO were both significantly less potent than the prototype anticonvulsant tiagabine. In contrast, *N*-methyl-*exo*-THPO was observed to be only slightly less potent than tiagabine against audiogenic seizures. In addition to their ability to reduce the tonic extension component of the audiogenic seizure, all four compounds were effective in blocking the clonic seizure (data not shown). The anticonvulsant activity of tiagabine and the *exo*-THPO family of compounds was correlated to their action as inhibitors of neuronal and astrocytic GABA uptake. Figure 4 shows the correlation between ED_{50} values for protection against audiogenic seizures in the Frings mouse and IC_{50} values for inhibition of GABA uptake into neurons and astrocytes. In the case of astrocytic GABA uptake, a strong correlation ($r^2 = 0.988$) was observed. In contrast, a weak correlation ($r^2 = 0.534$) was observed between the rank order of potency as anticonvulsants and that as inhibitors of neuronal GABA uptake.

TABLE 1

IC_{50} values for inhibition of GABA uptake in neurons, astrocytes, and cloned mouse GABA transporters expressed in HEK cells by *exo*-THPO, *N*-methyl-*exo*-THPO, *N*-ethyl-*exo*-THPO, and tiagabine

Neurons and astrocytes were cultured from mouse cerebral cortex and HEK 293 either transiently or stably expressing the cloned mouse GABA transporters GAT 1 to 4 were obtained as detailed under *Experimental Procedures*. IC_{50} values were determined at a fixed GABA concentration in micromolar and varying the inhibitor concentration. Subsequently, IC_{50} values were calculated as described under *Experimental Procedures*.

Compound	IC_{50} Values[a]					
	Neurons	Astrocytes	mGAT1	mGAT2	mGAT3	mGAT4
			μM			
exo-THPO	780 (505–1200)	250 (125–610)	1000	3000	>3000	>3000
N-Methyl-*exo*-THPO	405 (250–780)	48 (19–72)	450	>3000	>3000	>3000
N-Ethyl-*exo*-THPO	390 (301–510)	301 (160–710)	320	>1000	>1000	>1000
Tiagabine	0.36 (0.20–1.10)	0.18 (0.092–0.32)	0.8	300	>300	800

[a] 95% confidence interval in parentheses.

TABLE 2

Kinetic characterization of the inhibition of GABA transport in mGAT1 transfected HEK 293 cells by *exo*-THPO and *N*-methyl-*exo*-THPO

HEK 293 cells expressing mouse cloned GAT1 were obtained as detailed under *Experimental Procedures* and the kinetic analysis of GABA transport performed in the absence or presence of 500 or 1000 μM *exo*-THPO and 200 or 400 μM *N*-methyl-*exo*-THPO using a series of GABA concentrations, as described under *Experimental Procedures*. The V_{max} value in the absence of the inhibitor was 2.1 to 3.8 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} of protein. Statistically significant differences from the control (Student's *t* test) are indicated by asterisks (*$p < 0.01$; **$p < 0.001$).

Compound	K_m	V_{max}	K_i	Inhibition Type
	μM	%	μM	
Control	27 ± 4	100 ± 3		
exo-THPO (500 μM)	48 ± 3*	86 ± 3	641	Competitive
exo-THPO (1000 μM)	74 ± 3**	118 ± 3	575	Competitive
N-Methyl-*exo*-THPO (200 μM)	48 ± 3*	86 ± 3	256	Competitive
N-Methyl-*exo*-THPO (400 μM)	63 ± 3**	86 ± 2	301	Competitive

TABLE 3

Kinetic characterization of the inhibition of GABA transport in neurons and astrocytes by *exo*-THPO (EXO)

Cells were cultured from mouse cerebral cortex as detailed under *Experimental Procedures* and the kinetic analysis of GABA transport performed in the absence or presence of 900 μM (neurons) or 400 μM (astrocytes) EXO using a series of GABA concentrations as described under *Experimental Procedures.* The V_{max} values in the absence of the inhibitor were 3.20 ± 0.15 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} of protein and 0.13 ± 0.01 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} of protein in the neurons and astrocytes, respectively. Statistically significant differences from the control (Student's *t* test) are indicated by asterisks (*$p < 0.01$; **$p < 0.001$).

Cell Type	K_m		V_{max}		K_i	Type
	Control	EXO	Control	EXO		
	μM		%		μM	
Neuron	24 ± 3	56 ± 4**	100 ± 5	122 ± 5	680	Competitive
Astrocyte	8 ± 1	21 ± 3*	100 ± 6	123 ± 10	230	Competitive

Of the four compounds tested, tiagabine produced the greatest degree of impairment in the rotarod test. For example, at an i.c.v. dose of 48 nmol (approximately twice the ED_{50}), three of four mice tested failed the rotarod test. In comparison, none of the mice treated with 120 nmol of *N*-methyl-*exo*-THPO (approximately 2 times the ED_{50}), 170 nmol of *exo*-THPO (approximately the ED_{50}), or 450 nmol of *N*-ethyl-*exo*-THPO (approximately 3 times the ED_{50}) displayed any signs of rotarod impairment. Higher doses of *exo*-THPO and its alkylated derivatives could not be tested due to the limited solubility of the *exo*-THPO analogs tested. Although not conclusive, these findings seem to suggest that the protective index of all three *exo*-THPO analogs is equivalent to, or greater than, that of tiagabine.

Given the previously demonstrated lack of systemic activity of *N*-methyl-*exo*-THPO and *N*-ethyl-*exo*-THPO after i.p. administration (Falch et al., 1999), the *O*-pivaloyloxymethyl prodrug of *N*-methyl-*exo*-THPO (Fig. 1) was evaluated after i.p. and i.c.v. administration. When examined at its time to peak effect (Fig. 5, inset), the *N*-methyl-*exo*-THPO prodrug produced a dose-dependent inhibition of audiogenic seizures after i.p. administration (Fig. 5). At this time point, the calculated ED_{50} (95% confidence intervals) was 233 (191–273) μmol/kg. In addition, rotarod impairment was noted in 38% (3/8) of the mice tested at 306 μmol/kg. Interestingly, the *O*-pivaloyloxymethyl prodrug of *N*-methyl-*exo*-THPO was inactive after i.c.v. administration at toxic doses of 61 μmol (data not shown).

Discussion

The results of the present investigation confirm previous findings that the bicyclic isoxazole *exo*-THPO and its *N*-methylated and *N*-ethylated analogs are potent inhibitors of neuronal and glial GABA uptake (Falch et al., 1999). In addition, the IC_{50} values reported herein for *exo*-THPO, *N*-methyl-*exo*-THPO, *N*-ethyl-*exo*-THPO, and tiagabine were found to be essentially identical to previously reported values (Braestrup et al., 1990; Falch et al., 1999). The present study extends these previous findings by demonstrating that *exo*-

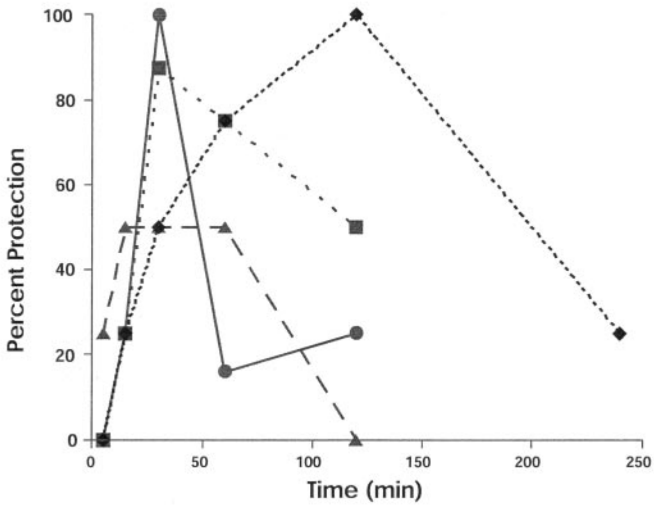

Fig. 2. Time-effect relationship for the anticonvulsant activity of *exo*-THPO (128 nmol; solid circle, solid line), *N*-methyl-*exo*-THPO (120 nmol; solid square, dotted line), *N*-ethyl-*exo*-THPO (114 nmol; solid triangle, dashed line), and tiagabine (49 nmol; solid diamond, dotted line) after i.c.v. administration to Frings AGS mice. Each test substance was injected i.c.v. into the lateral ventricle of awake mice (n = 4–8 mice/time point) in a total volume of 5 μl using a 10-μl Hamilton syringe. At various times after dosing, individual mice were challenged with a high-intensity sound stimulus. The percentage of animals at each time point not displaying a tonic extension seizure was recorded and plotted as a function of time.

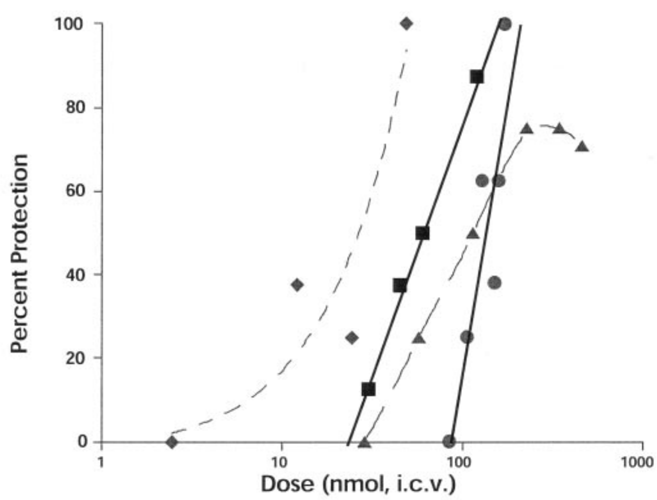

Fig. 3. Dose-response relationship for the anticonvulsant action of *exo*-THPO (solid circles, solid line), *N*-methyl-*exo*-THPO (solid squares, solid line), *N*-ethyl-*exo*-THPO (solid triangle, dashed line), and tiagabine (solid diamond, dashed line) after i.c.v. administration to Frings AGS mice (n = 8–13 mice/dose level). Results are expressed as a percentage of animals protected against a tonic extension seizure induced by a high-intensity sound stimulus. Anticonvulsant activity was quantitated at the time to peak effect as defined by the time-response study shown in Fig. 2.

THPO and its two *N*-alkylated analogs are highly selective inhibitors of the cloned GAT1 transporter being inactive at GAT2–4. Furthermore, all three isoxazoles were found to exert their inhibitory effect through a competitive interaction with the GABA transporter. These findings are similar to those reported for the bicyclic isoxazole THPO, which is isomeric with *exo*-THPO (Fig. 1; Bolvig et al., 1999). Importantly, the present study further confirms the marked glial selectivity of *N*-methyl-*exo*-THPO. This is particularly interesting in light of the finding that this compound was found to

TABLE 4

Effect of *exo*-THPO and its *N*-alkylated analogs on audiogenic seizures following i.c.v. administration to Frings AGS mice

Frings audiogenic seizure-susceptible mice were treated with varying i.c.v. doses of *exo*-THPO, *N*-methyl-*exo*-THPO, *N*-ethyl-*exo*-THPO, and tiagabine. At the time to peak effect of each inhibitor, individual mice were challenged with a high-intensity auditory stimulus. Mice not displaying tonic hind-limb extension were considered protected. The ED_{50} and corresponding 95% CI values were calculated by probit analysis of the dose-response data.

	Anticonvulsant Activity (nmol i.c.v.)	
	ED_{50}	95% CI*
exo-THPO	136	115–155
N-Methyl-*exo*-THPO	59	41–94**
N-Ethyl-*exo*-THPO	155	88–255
Tiagabine	22	11–36

* 95% confidence interval.
** Falch et al. (1999).

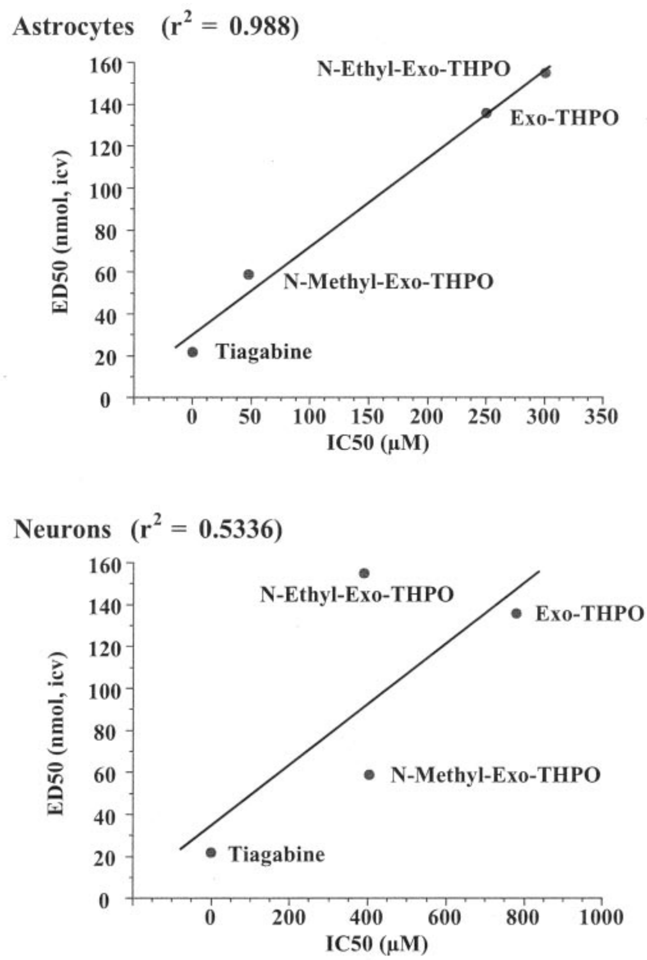

Fig. 4. Correlation between the ED_{50} for protection against sound-induced seizures and the IC_{50} for GABA uptake into astrocytes (A) and neurons (B) for *exo*-THPO, *N*-methyl-*exo*-THPO, *N*-ethyl-*exo*-THPO, and tiagabine. ED_{50} values for tiagabine, *exo*-THPO, and its analogs were calculated by probit analysis of the dose-response data plotted in Fig. 3 and plotted against the IC_{50} values obtained from GABA inhibition studies conducted with neuronal and glial cells grown and maintained in tissue culture.

be highly selective for GAT1. The fact that both neurons and glial cells express GAT1 is not sufficient to explain the cellular selectivity of *N*-methyl-*exo*-THPO. At the present time, no exact explanation is obvious. However, it may be hypothesized that cooperative interactions may exist among the

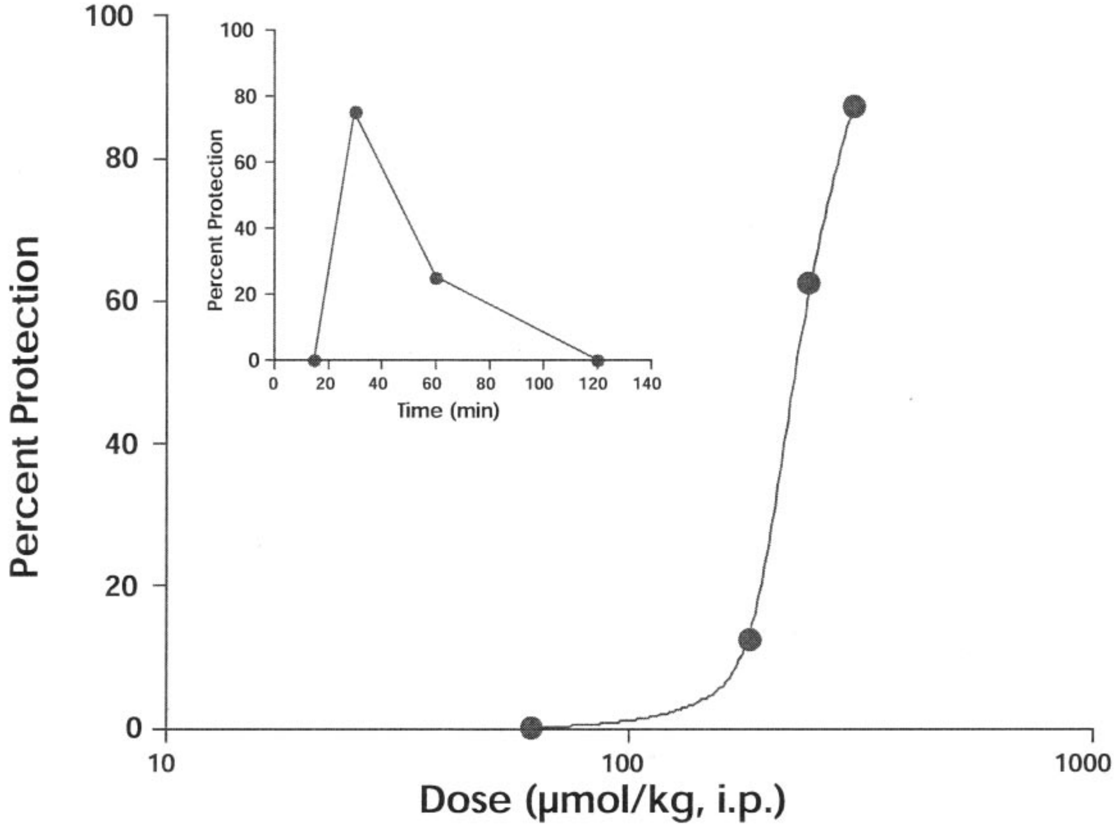

Fig. 5. Time- and dose-effect relationship for the anticonvulsant activity of the *O*-pivaloyloxymethyl prodrug of *N*-methyl-*exo*-THPO after i.p. administration to Frings AGS mice. Results obtained from the time-response study are plotted in the inset as a percentage of mice not displaying a tonic extension seizure at the various times (n = 4 mice/time point) tested. For the dose-response study, groups of eight mice were treated with varying doses of the *O*-pivaloyloxymethyl prodrug and tested at the time of peak anticonvulsant effect (i.e., 30 min) for protection against sound-induced tonic extension seizures.

different GABA transporters, several of which may be expressed on the same cell (Gadea and Lopez-Colome, 2001; Schousboe and Kanner, 2002). Thus, it is conceivable that such an interaction may alter the pharmacological properties of the transporters, thereby explaining the unexpectedly high potency of *N*-methyl-*exo*-THPO as an inhibitor of GABA uptake in astrocytes. It can, however, not be excluded that transporters so far not cloned and with unknown pharmacological properties may exist.

Both *exo*-THPO and its two *N*-alkylated analogs were found to possess potent anticonvulsant activity when administered i.c.v. to Frings AGS mice. Moreover, for all three compounds tested, the anticonvulsant activity was observed at i.c.v. doses devoid of behavioral impairment. Based on previous findings in the isoniazid seizure model wherein it was demonstrated that the anticonvulsant activity of *N*-methyl-*exo*-THPO was restricted to the *R*-enantiomer (Falch et al., 1999), it is likely that the anticonvulsant activity of *N*-methyl-*exo*-THPO in the Frings mouse is also stereoselective. This conclusion is supported by the previous demonstration that inhibition of GABA uptake is more pronounced by the *R*-enantiomer rather than the *S*-enantiomer (Falch et al., 1999). Interestingly, *N*-methyl-*exo*-THPO was the most potent of the three analogs tested followed by *exo*-THPO and *N*-ethyl-*exo*-THPO. This rank order of potency was found to correlate very well with the rank order of inhibition of GABA uptake found in astrocytes (r^2 = 0.988). In contrast, a weak correlation was observed between inhibition of neuronal GABA uptake and anticonvulsant activity of *exo*-THPO, its two alkylated analogs, and tiagabine (r^2 = 0.534). Although preliminary in nature, these findings provide theoretical support for the potential therapeutic utility of a highly selective glial GABA uptake inhibitor. Furthermore, as mentioned above pure neuronal selective GABA uptake inhibitors display proconvulsant activity, presumably as a result of their ability to deplete the transmitter GABA pool (Schousboe et al., 1983; Gonsalves et al., 1989b). In contrast, glial selective inhibitors increase both the extrasynaptic and synaptosomal levels of GABA without disrupting GABA metabolism (Wood et al., 1983; Juhasz et al., 1997).

Tiagabine was also found to be a potent anticonvulsant in the Frings AGS mouse. This finding is in keeping with its previously demonstrated anticonvulsant activity (Suzdak and Jansen, 1995). Interestingly, tiagabine was less potent as an anticonvulsant than might be predicted by its ability to inhibit GABA uptake. For example, compared with *exo*-THPO and its *N*-alkylated analogs, tiagabine was between 100- and 1000-fold more potent as an inhibitor of GABA uptake; however, it was only 2.5- to 7-fold more potent as an anticonvulsant. At the present time, there is no obvious explanation for this interesting dichotomy. However, it should be noted that the actual concentration of tiagabine at the site of action could be lower than predicted from the amount injected because the onset of action of tiagabine was delayed considerably from the time of injection, a phenomenon not seen for the *exo*-THPO family of compounds.

Previous investigations have shown the *O*-pivaloyloxymethyl prodrug of *N*-methyl-*exo*-THPO to be active against isoniazid-induced seizures (Falch et al., 1999). The present study found this prodrug to be active against audiogenic seizures after i.p. administration to Frings mice. After i.p. administration, this prodrug displayed a time- and dose-dependent inhibition of audiogenic seizures that is thought to result from the cleavage of the pivaloyloxymethyl from the parent compound upon entrance into the CNS. As noted in the time course study (Fig. 5, inset) a dramatic decrease in anticonvulsant activity was observed 60 min after i.p. administration. This differs from the rather long duration of action observed after i.c.v. administration of the parent compound (i.e., more than 2 h). This may be explained in part by a relatively rapid peripheral metabolism of the prodrug before penetration of the BBB. By decreasing the total amount available, less of the prodrug is therefore available for cleavage of the pivaloyloxymethyl group upon entrance into the CNS. Interestingly, the direct i.c.v. injection of the prodrug into the ventricular compartment of the brain failed to exert an anticonvulsant action. This apparent discrepancy is explained in part by the presence of marked toxicity after i.c.v. administration of the prodrug that was not observed after i.c.v. administration of the parent compound. Nonetheless, the results of the present study when coupled with previous findings in the isoniazid seizure model (Falch et al., 1999) confirm the validity of the prodrug approach and may represent a viable formulation for the systemic administration of *N*-methyl-*exo*-THPO and other bicyclic isoxazole derivatives. Alternatively, more lipophilic analogs could be developed to enhance penetration of the BBB while at the same time preserving the glial selectivity of *N*-methyl-*exo*-THPO.

Surprisingly, *N*-methyl-*exo*-THPO was found to be only 2-fold less potent than tiagabine as an anticonvulsant. This observation, taken together with the finding that *N*-methyl-*exo*-THPO-treated animals perform better than tiagabine-treated mice in the rotarod toxicity test, suggests that this new class of GABA transport inhibitors may represent a better tolerated alternative to tiagabine as antiseizure agents. Clearly, testing of these compounds is at a very early stage of development and other seizure models must be evaluated before conclusions can be reached regarding the overall therapeutic potential of this class of compounds. Nonetheless, the results of the present investigation support the continued evaluation of this class of compounds as anticonvulsants and development of more lipophilic analogs.

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