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GABA transporters as drug targets for modulation of GABAergic activity

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Abstract

The identification and subsequent development of the GABA transport inhibitor tiagabine has confirmed the important role that GABA transporters play in the control of CNS excitability. Tiagabine was later demonstrated to be a selective inhibitor of the GABA transporter GAT1. Although selective for GAT1, tiagabine lacks cell type selectivity and is an equipotent inhibitor of neuronal and glial GAT1. To date, four GABA transporters have been cloned, i.e., GAT1-4. The finding that some of these display differential cellular and regional expression patterns suggests that drugs targeting GABA transporters other than GAT1 might offer some therapeutic advantage over GAT1 selective inhibitors. Furthermore, it is particularly interesting that several recently defined GABA transport inhibitors have been demonstrated to display a preferential selectivity for the astrocytic GAT1 transporter. That cellular heterogeneity of GAT1 plays a role in the control of CNS function is confirmed by the demonstration that inhibition of astrocytic GABA uptake is highly correlated to anticonvulsant activity. At the present time, a functional role for the other GABA transporters is less well defined. However, recent findings have suggested a role for the mouse GAT2 (homologous to the human betaine transporter) in the control of seizure activity. In these studies, the non-selective GAT1 and mouse GAT2 transport inhibitor EF1502 (*N*-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-hydroxy-4-(methylamino)-4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-ol) was found to exert a synergistic anticonvulsant action when tested in combination with the GAT1 selective inhibitors tiagabine and LU-32-176B (*N*-[4,4-bis(4-fluorophenyl)-butyl]-3-hydroxy-4-amino-4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-ol). Additional studies will be required to define a role for the other GABA transporters and to further identify the functional importance of their demonstrated cellular and regional heterogeneity. A summary of these and other issues are discussed in this brief review.

Keywords: GAT1-4; BGT1; Epilepsy; Antiepileptic drug; Animal seizure models

1. Introduction

γ-Aminobutyric acid (GABA) has been known to be present in plants and bacteria for almost a hundred years [1,2] and the decarboxylase catalyzing its formation from glutamate was described in bacteria early on [3]. Its presence in brain tissue together with its biosynthetic enzyme glutamate decarboxylase (GAD) was described simultaneously in three independent publications in 1950 [4–6]. One of the first indications that GABA could play an important role in brain function came from studies with the convulsant hydrazides which inhibit its synthesis [7,8] and the demonstration by Bazemore et al. [9] that a factor in brain extracts that caused an inhibitory action at the crayfish stretch receptor was actually GABA. Further elegant electrophysiological studies soon confirmed that GABA was indeed an important inhibitory neurotransmitter [10,11].

Any neurotransmission system is dependent upon an effective mechanism by which the action of the transmitter can be inactivated. In case of GABA, it is clear that a metabolic process is unlikely to be responsible for this since its catabolic enzyme GABA transaminase is located intracellularly in the inner membrane of mitochondria [12,13]. Reports describing the capacity of brain slices and homogenates and cell preparations to accumulate radioactively labelled GABA [14–16] have underlined

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the importance of active transmembrane transport of GABA for its inactivation. Numerous studies describing GABA transport in brain cell preparations (see Schousboe [17]) have confirmed that GABAergic nerve endings as well as astrocytes play an important role in this process. Since the fate of GABA may be completely different depending on whether the site of uptake is neuronal or astrocytic one should consider the functional consequences. It appears that the majority of released GABA is transported back into the presynaptic GABAergic nerve ending whereas a much smaller fraction is taken up by astrocytes surrounding the synapse [18]. GABA taken up into the nerve ending is likely to be reutilized as a transmitter by inclusion in synaptic vesicles [19] whereas that taken up by astroglial cells may be lost by metabolism, although a fraction may be available to neurons in the form of glutamine which may be transferred from the astrocytic to the neuronal compartment [18,20].

In order to elucidate the functional roles of these transport processes it is necessary to obtain detailed knowledge about the anatomical distribution of GABA transporters as well as their pharmacological properties. The cloning of four GABA transporters (mouse nomenclature GAT1-4) has allowed such studies to be performed over the past 15 years. The present review presents results of pharmacological characterization of GABA transporters and provides a discussion of the functional role of these transporters for the control of epilepsy.

2. Materials and methods

2.1. Neural cell cultures

Primary cultures of GABAergic neurons and cerebral cortical astrocytes were prepared as detailed by [21,22]. Briefly, cell suspensions from cerebral cortices isolated from 15-day-old mouse embryos or newborn mice were prepared by trypsinization or mechanical trituration and seeded in culture flasks to produce neuronal and astrocytic cultures, respectively.

2.2. Cloned transporters expressed in cell lines

The cDNAs of the cloned mouse GABA transporters GAT1-4 (GAT2 corresponds to the betaine transporter BGT-1) obtained from Dr. Nathan Nelson, Tel Aviv University, Tel Aviv, Israel, were used to produce subclones suitable for transfection into HEK 293 cells as detailed in White et al. [23].

2.3. GABA transport assay

Cultured neural cells or HEK 293 cells expressing the cloned GABA transporters were used to determine IC_{50} values or K_i values for the inhibition of GABA transport by a variety of structural analogs of GABA. The uptake of [^{3}H]GABA either at 1 μM (IC_{50} values) or a concentration range (K_i values) was determined in the absence or presence of the various GABA transport inhibitors at varying concentrations [23]. Calculation of IC_{50} or K_i values was performed as detailed by White et al. [23].

2.4. Assessment of anticonvulsant activity

The ability of selected inhibitors of GABA transport to protect against seizures was tested in audiogenic seizure (AGS)-susceptible Frings mice as detailed by White et al. [23]. The GABA analogs were tested by intracerebroventricularly (i.c.v.) administration into the lateral ventricle of conscious mice using a 10 μL Hamilton syringe. The number of mice not displaying tonic hindlimb extension was recorded for each dose evaluated and the median effective dose (ED_{50}) and 95% confidence intervals calculated by probit analysis.

3. Results

3.1. GABA analogs of restricted conformation

Early structure–activity studies of GABA transport and GABA receptor binding led to the identification of chemical structures capable of specifically interacting with receptor binding and transport sites, respectively [24]. Fig. 1 shows the chemical structures of GABA analogs that have served as lead compounds for the development of GABA transport inhibitors. Among these, nipecotic acid and guvacine have been used to produce hundreds of analogs mainly with lipophilic side chains linked to the nitrogen atom in the pyridine ring. This class of analogs was originally developed to circumvent the problem of penetration of the hydrophilic parent compound at the blood–brain barrier (BBB) [25,26]. This strategy for development of clinically active antiepileptic drugs resulted in the synthesis and preclinical development of tiagabine [27,28] which is now in clinical use [29]. THPO (4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol), which is a bioisostere of nipecotic acid has served as a lead structure to develop *exo*-THPO (4-amino-4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-ol) and a large number of lipophhilic analogs thereof. Several of these compounds are discussed below.

3.2. Pharmacology of GABA transport in neurons and astrocytes

Inhibitory constants for GABA transport in neurons and astrocytes of several GABA analogs derived from nipecotic acid or THPO are provided in Table 1. It is worth noting that in contrast to nipecotic acid and guvacine, which are substrates for the GABA transporters, the

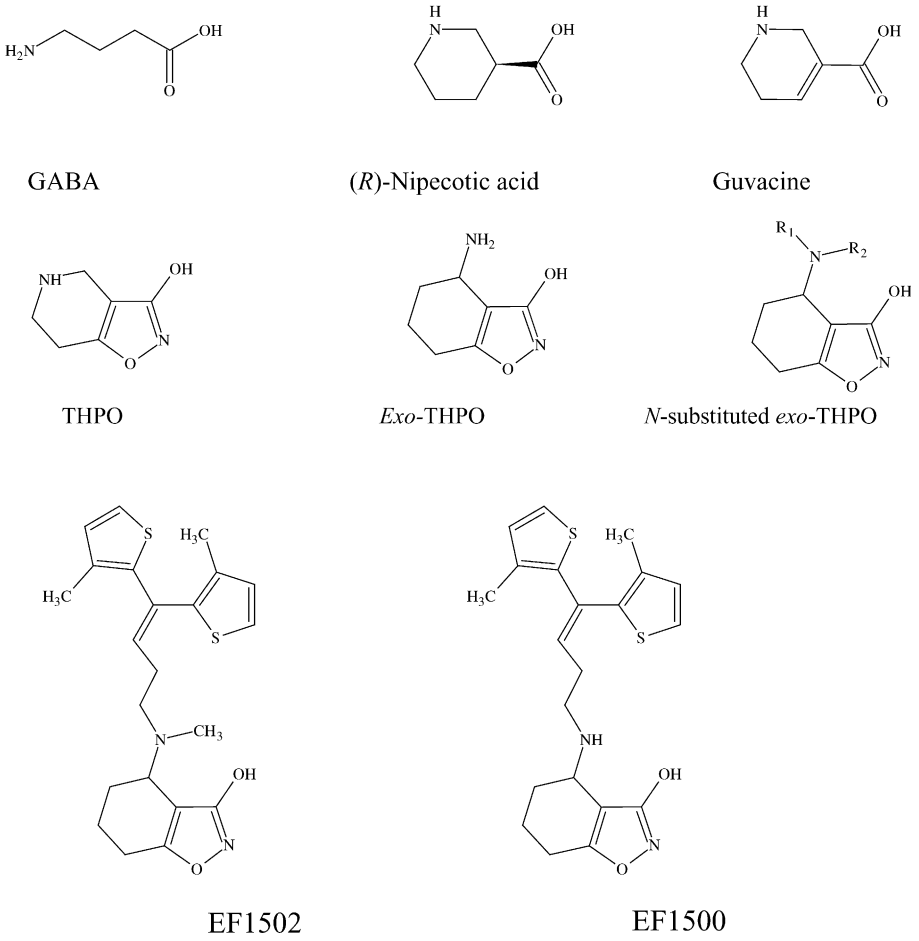


Fig. 1. Chemical structures of GABA and of key selective GABA-transport inhibitors of restricted conformation.

bicyclic isoxazole bioisostere THPO is not a substrate [30,31]. Furthermore, none of the lipophilic analogs studied, regardless of the nature of the GABA mimetic moiety of the molecule, were found to act as a substrate for the transporter.

A large number of analogs of *exo*-THPO have recently been investigated as inhibitors of GABA transport in neurons and astrocytes [23,32–34]. Some of the available inhibitory constants have been summarized in Table 2. It can be seen that some of these compounds can be considered selective inhibitors of astroglial GABA transport having a 5–10-fold higher affinity for the glial transport system compared to that in neurons. As shall be discussed below, this may be of functional importance when considering their anticonvulsant activity. Moreover, the potency of this class of GABA analogs is greatly improved by adding a lipophilic side chain at the primary amine nitrogen, a feature shared by the lipophilic analogs of nipecotic acid and THPO (Table 1). Cell-selectivity might have been anticipated had differences in their ability to inhibit GABA uptake into HEK cells expressing the cloned GABA transporters been observed, since each of these transporters have different distribution patterns in neurons and glia [35–37]. However, as will be discussed below, the above scenario does not appear to reflect this line of reasoning.

Table 1
IC_{50} or K_i values for inhibition by nipecotic acid or THPO analogs of GABA transport in neurons and astroglial cells

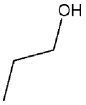
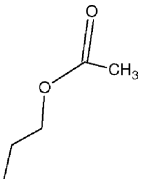
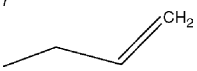
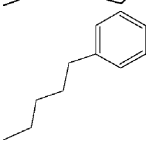
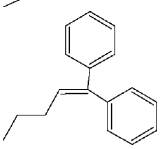
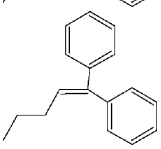
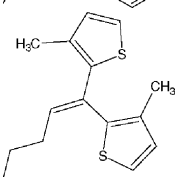
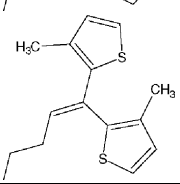
Inhibitor	IC_{50} or K_i (μM)		Substrate
	Neurons	Astrocytes	
GABA	8	32	Yes
Nipecotic acid	12	16	Yes
Guvacine	32	29	Yes
THPO	501	262	No
THAO	487	258	No
DPB–nipecotic acid	1.3	4.2	No
DPB–guvacine	4.9	4.2	No
Tiagabine	0.45	0.18	No
DPB–THPO	38	26	No
DPB–THAO	9	3	No

Original data are from Sarup et al. [36] and references therein.

3.3. Pharmacology of cloned GABA transporters

The classical GABA transport inhibitor nipecotic acid inhibits all cloned GABA transporters except the betaine (BGT-1)/GABA transporter (GAT2-mouse) with IC_{50}

Table 2
IC_{50} values for inhibition by exo-THPO and its N-substituted analogs of GABA transport in neurons and astrocytes

Inhibitor	*N*-substitution		IC_{50} (M)	
	R_1	R_2	Neurons	Astrocytes
exo-THPO	H	H	780	250
N-Methyl–		H	405	48
N,*N*-Dimethyl–			>1000	>1000
N-Ethyl–		H	390	301
N-2-Hydroxyethyl–		H	300	200
N-Aucetyloxyethyl–		H	300	18
N-Allyl–		H	220	73
N-Phenylbutyl–		H	100	15
N-DPB–		H	1.4	0.6
N-DPB-*N*-methyl–			5	2
EF1500		H	4	2
EF1502			2	2

Original data from Sarup et al. [36] and White et al. [33].

values of 25–100 μM [35,38]. β-Alanine, on the other hand, inhibits only GAT3 and GAT4 (mouse) corresponding to GAT-2 and GAT-3 in the rat [35,38]. As shown in Table 3, a large number of lipophilic analogs of nipecotic acid also inhibit GAT1, GAT3 and GAT4 but have no affinity for GAT2 or BGT-1. The only exception is the *N*-trimethoxyphenyl-t-butoxyethyl-nipecotic acid (SNAP-5114) which inhibits GAT2, GAT3 and GAT4 with higher affinity than GAT1 [35,39]. Other inhibitors which

Table 3
Effect of GABA analogs of restricted conformation on GABA transport mediated by GAT1-4

Compound	IC_{50} (M)			
	GAT1	GAT2/BGT1	GAT3	GAT4
Nipecotic acid	24	>1000	113	159
THPO	1300	3000	800	5000
exo-THPO	1000	3000	>3000	>3000
N-DPB-nipecotic acid	0.6	210	550	4390
Tiagabine	0.8	300	>300	800
SNAP-5114	>30	22	20	6.6
N-DPB-THPO	30	200	>300	>1000
N-Methyl-*exo*-THPO	450	>3000	>3000	>3000
N-Ethyl-*exo*-THPO	320	>1000	>1000	>1000
N-Acetyloxyethyl-*exo*-THPO	550	>1000	>1000	>1000
N-Phenylbutyl-*exo*-THPO	7	>500	>1000	>1000
N-DPB-*exo*-THPO	6	100	>100	>100
N-DPB-*N*-methyl-*exo*-THPO				
EF1500	3	130	>100	>100
EF1502	4	22	>300	>300
NNC-05-2045	27	1.6	14	6.1
NNC-05-2090	19	1.4	41	15

Original data from Sarup et al. [36,38], White et al. [23,33], Thomsen et al. [39], and Borden [35].

are not strictly GABA analogs have recently been reported to inhibit all four cloned GABA transporters (Table 3) generally with somewhat higher affinity for GAT2 and GAT4 than for GAT1 and GAT3 [39]. As discussed below, these compounds may be of interest as anticonvulsants.

Among the second generation GABA transport inhibitors developed using THPO as a lead structure (i.e., *exo*-THPO analogs), some of which exhibited selectivity for astroglial GABA transport (Table 2), none have been found to inhibit GABA transport mediated by GAT3 and GAT4 [34,36]. All of these compounds regardless of the nature of the constituents at the amino group have been found to inhibit GAT1 [34,36,38]. The affinity for the transporter has been found to increase with more pronounced lipophilicity of the constituents on the amino nitrogen [34,36]. In the light of this, it was quite surprising to find that one particular analog of *exo*-THPO, EF1502 (*N*-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-hydroxy-4-(methylamino)-4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-ol) the structure of which is shown in Fig. 1, turned out to be essentially equipotent as an inhibitor of GAT1 and GAT2 mediated GABA transport (Table 3). Interestingly, EF1500 (*N*-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-hydroxy-4-amino-4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-ol) having the same structure but lacking the methyl group on the amino nitrogen (Fig. 1) had no affinity for GAT2 but inhibited GAT1-mediated GABA transport with the same potency as EF1502 (Table 3). Both compounds inhibited neuronal and glial GABA uptake equipotently. As will be discussed below EF1502 has been found to exhibit a highly unusual profile with regard to its protection against acute evoked and kindled seizures.

Table 4
Anticonvulsant activity of tiagabine and exo-THPO and its N-substituted analogs

Compound	Anticonvulsant activity	
	ED_{50} (nmol i.c.v.)	95% CI
Tiagabine	22	11–36
exo-THPO	136	115–155
N-Methyl-*exo*-THPO	59	41–94
N-Ethyl-*exo*-THPO	155	88–255
EF1502	84	66–114

Original data from White et al. [23] or from experiments performed in this study (EF1502).

3.4. Anticonvulsant activity of GABA transport inhibitors

The ability of GABA transport inhibitors to prevent audiogenic seizures or chemically induced seizures was demonstrated more than 20 years ago [40–44]. As shown in Table 4, *exo*-THPO and selected *N* substituted analogs have comparable anticonvulsant activity to the clinically active antiepileptic drug tiagabine. Due to penetration problems at the BBB only the lipophilic analogs of *exo*-THPO could be tested by peripheral administration. It is of particular interest that EF1502 has been shown to exert a synergistic anticonvulsant activity when administered together with tiagabine or another liphophilic GAT1-selective GABA-transport inhibitor LU-32-176B (*N*-[4,4-bis(4-fluorophenyl)-butyl]-3-hydroxy-4-amino-4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-ol) [33]. The possibility that this synergistic action of EF1502 is related to its ability to inhibit GAT2 mediated GABA transport is discussed below.

4. Discussion

4.1. Pharmacological profile of transporters

It may appear enigmatic that *exo*-THPO and some of its *N* substituted analogs have been found to be at least partly selective for astroglial GABA transport with less affinity for neuronal GABA transport given that they are active as inhibitors of GAT1 yet have no affinity for the other cloned GABA transporters [36–38]. Whether cooperativity may exist at the cellular level between individual transporters in oligomeric transporter complexes allowing for pharmacological properties different from those of the individual transporters remains to be elucidated. The possibility that such oligomeric transporters could exist is supported by several reports concerning glutamate and monoamine transporters (see Danbolt [45]).

Another interesting feature of the inhibitors of GAT1 is the finding that while nipecotic acid is a substrate for the transporter [30], its bioisosteric analog THPO is not [31]. At the present time it is not known if *exo*-THPO can act as a substrate; however, it is known to act as a competitive inhibitor of GABA transport in these preparations [38]. Since THPO is also a competitive inhibitor [46,47] but not a substrate, it may well be that *exo*-THPO is not a substrate. It should be noted that the lipophilic analog of *exo*-THPO, EF1502 has been shown to be a non-competitive inhibitor of GABA transport both at GAT1 and GAT2 and not to act as substrates for the GABA transporters expressed in cultured GABAergic neurons [33]. Likewise, lipophilic analogs of nipecotic acid and guvacine have been shown to not act as a substrate for GABA transport in neurons and astrocytes [48].

The finding that some of these lipophilic analogs of *exo*-THPO are non-competitive inhibitors of GABA transport [33,38] may indicate that there is a binding site on the transporter different from the substrate recognition site which is likely to be involved in binding of the competitive lipophilic analogs of, e.g., nipecotic acid. Such a binding model has previously been proposed for tiagabine [27]. More detailed structure–activity studies may be required to fully elucidate these questions.

4.2. Anticonvulsant activity of transport inhibitors

As mentioned above it has been known for over 20 years that inhibitors of GABA transport have anticonvulsant activity. This is historically the reason why an emphasis was placed on development of analogs which could penetrate the BBB as the structurally restricted GABA analogs originally available (nipecotic acid, guvacine, and THPO) or later developed (*exo*-THPO) do not pass the BBB (e.g., Schousboe et al. [49]). The general strategy has relied on lipophilic analogs [26] or prodrugs [40]. The subsequent development of tiagabine as a clinically effective antiepileptic drug [29] has proven this strategy to be correct.

It has been somewhat enigmatic for a number of years why some of the less potent inhibitors of GABA transport such as THPO and *exo*-THPO have proven highly efficatious and unexpectedly potent as anticonvulsants when administered i.c.v. [23,50]. A recent analysis of the correlation between their potency as anticonvulsants and their potency as GABA transport inhibitors in neurons and astrocytes, respectively clearly showed a much better correlation with inhibition in astrocytes than in neurons [23]. This underlines the suggestion put forward a number of years ago [43] that since astrocytic GABA transport would likely lead to a drain on the synaptic GABA pool, selective inhibition of this transport system would facilitate reuptake into nerve endings leading to an enhanced GABAergic tone protecting against epileptic seizures. This line of reasoning leads to considerations concerning the anatomical distribution of GABA transporters. Numerous studies have shown that while GAT1 is preferentially located on neuronal elements with less expression on astrocytes, GAT2/BGT1, GAT3 (rat GAT-2) and GAT4 (rat GAT-3) are preferentially located on non-neuronal

elements (see Gadea and Lopez-Colome [51] and Sarup et al. [36]). As discussed in detail elsewhere this has led to the suggestion that inhibitors of GABA transport mediated by transporters other than GAT1 may be interesting as anticonvulsants [36,52,53]. Indeed, it has been experimentally demonstrated that such inhibitors do possess anticonvulsant activity [33,52–54]. The synergistic anticonvulsant activity of EF1502 when administered together with tiagabine strongly suggests that GAT2/BGT1 inhibitors may be particularly attractive as anticonvulsants thereby making the betaine transporter an important target for anticonvulsant drugs [33,53]. Perhaps, more importantly is the observation that the combination of EF1502 with tiagabine did not increase the behavioural toxicity of either drug alone. From these studies, it would appear that selective inhibitors of the GAT2/BGT1 transporter may offer some advantage over pure GAT1 selective inhibitors. Current efforts in our laboratory are aimed at producing GABA analogs with a selective action on GAT2/BGT1. Clearly, the successful clinical development of a GAT2/BGT1 inhibitor will be required before this hypothesis can be either confirmed or refuted.

Acknowledgements

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