available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

Inhibition of rabbit brain 4-aminobutyrate transaminase by some taurine analogues: A kinetic analysis

Lorenzo Ricci [a], *Maria Frosini* [a,*], *Nicola Gaggelli* [b], *Gianni Valensin* [b], *Fabrizio Machetti* [c], *Giampietro Sgaragli* [a], *Massimo Valoti* [a]

[a] *Dipartimento di Scienze Biomediche, Sezione di Farmacologia, Università di Siena, viale A. Moro 2, lotto C, 53100 Siena, Italy*
[b] *Dipartimento di Chimica e Centro NMR, Università di Siena, viale A. Moro 2, 53100 Siena, Italy*
[c] *Istituto di Chimica dei Composti Organo Metallici-CNR c/o Dipartimento di Chimica Organica 'U. Schiff', Università di Firenze, Firenze, Italy*

ARTICLE INFO

Article history:
Received 22 December 2005
Accepted 6 February 2006

Keywords:
GABA
4-Aminobutyrate transaminase
Vigabatrin
Taurine analogues
4-Aminobutyrate transaminase inhibitors

Abbreviations:
AEP, 2-aminoethylphosphonic acid
ANSA, 2-aminobenzenesulfonate
ATAHS, (±)*trans*-2-acetylaminocyclohexane sulfonic acid
GABA-T, 4-aminobutyrate transaminase (EC 2.6.1.19)
PSA, (±)piperidine-3-sulfonic acid
TA, Taurine analogues
VIGA, 4-amino-5-hexenoic acid or γ-vinyl GABA

ABSTRACT

The use of the antiepileptic drug, 4-aminobutyrate transaminase (GABA-T) inhibitor vigabatrin (VIGA), has been recently cautioned because it is associated to irreversible field defects from damage of the retina. Since novel GABA-T inhibitors might prove useful in epilepsy or other CNS pathologies as VIGA substitutes, the aim of the present investigation was to characterize the biochemical properties of some taurine analogues (TA) previously shown to act as GABA-T inhibitors. These include (±)piperidine-3-sulfonic acid (PSA), 2-aminoethylphosphonic acid (AEP), (±)2-acetylaminocyclohexane sulfonic acid (ATAHS) and 2-aminobenzenesulfonate (ANSA). Kinetic analysis of the activity of partially purified rabbit brain GABA-T in the presence of VIGA and TA showed that PSA and AEP caused a linear, mixed-type inhibition (K_i values 364 and 1010 μM, respectively), whereas VIGA, ANSA and ATAHS behaved like competitive inhibitors (K_i values 320, 434 and 598 μM, respectively). Among the compounds studied, only VIGA exerted a time-dependent, irreversible inhibition of the enzyme, with K_i and k_{inact} values of 773 μM and 0.14 min^{-1}, respectively. Furthermore, the ability of VIGA and TA to enhance GABA-ergic transmission was assessed in rabbit brain cortical slices by NMR quantitative analysis. The results demonstrate that VIGA as well as all TA promoted a significant increase of GABA content. In conclusion, PSA, ANSA and ATAHS, reversible GABA-T inhibitors with K_i values close to that of VIGA, represent a new class of compounds, susceptible of therapeutic exploitation in many disorders associated with low levels of GABA in brain tissues.

* *Corresponding author*. Tel.: +39 0577234441; fax: +39 0577234446.
E-mail address: frosinim@unisi.it (M. Frosini).
0006-2952/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2006.02.007

1. Introduction

γ-Aminobutyric acid (GABA) is one of the most important inhibitory neurotransmitters and its concentration in the brain is regulated by two pyridoxal 5′-phosphate (PLP)-dependent enzymes: L-glutamic acid decarboxylase, which catalyzes the conversion of L-glutamate to GABA, and 4-aminobutyrate transaminase (GABA-T, EC 2.6.1.19), which deaminates GABA to succinic semialdehyde (SSA) [1]. GABA-T, a homodimer with each subunit containing an active site, belongs to the subfamily II of the α-family of PLP-dependent enzymes [2]. Its mechanism of catalysis is well known: GABA is deaminated to succinic semialdehyde, thereby converting the PLP cofactor to its pyridoxamine 5′-phosphate (PMP) form [3]. In the reverse half-reaction, the enzyme is regenerated by transamination of α-ketoglutarate to glutamate.

A decrease in GABA brain levels has been implicated not only in the symptoms associated with epilepsy [4,5] but also in several other neurological diseases such as Huntington's chorea [6], Parkinson's disease [7], Alzheimer's disease [8], brain ischemia [9] and tardive dyskinesia [10]. Oral or parenteral administration of GABA is not feasible, as it cannot overcome the blood–brain barrier under normal conditions. However, a drug that has direct access to the brain and there selectively inhibits or inactivates GABA-T should be effective to increase brain concentrations of GABA. Several competitive inhibitors of GABA-T, particularly compounds with a backbone structure similar to GABA, and a variety of mechanism-based inactivators [11] of GABA-T exhibit anticonvulsant activity in experimental settings [12,13]. Vigabatrin (4-amino-5-hexenoic acid or γ-vinyl GABA) (VIGA) one of the latter compounds, has been shown to be effective for treating epilepsies that are resistant to other antiepileptic drugs [14]. Unfortunately, patients have suffered from an irreversible loss of their peripheral visual field upon treatment with this drug and their central visual function has also been irreversibly altered. However, the molecular mechanism of VIGA-attributed retinal toxicity is still unclear [15–17] and reports of visual dysfunction in patients with epilepsy treated with other anticonvulsant drugs raise the possibility that such deficits might be a relatively common side effect of anticonvulsant treatment or even a feature of the natural history of epilepsy itself [18,19]. Consequently, novel GABA-T inhibitors might prove useful not only as novel therapeutic agents in epilepsy or other CNS pathologies, but also as pharmacological tools to investigate whether visual field constriction is restricted to VIGA treatment alone. A recent study performed in this laboratory has demonstrated that some structural taurine analogues (TA) were able to inhibit rabbit brain GABA-T with IC_{50} values close to that of VIGA [20]. The present report describes their features as GABA-T inhibitors. (±)3-Piperidinsulfonic acid (PSA), 2-aminoethylphosphonic acid (AEP), 2-aminobenzenesulfonate (ANSA) and (±)*trans*-2-acetylaminocyclohexane sulfonic acid (ATAHS) behaved like reversible, not time-dependent GABA-T inhibitors with K_i values close to that of VIGA. They might represent a new class of GABA-T inhibitors, susceptible of therapeutic exploitation in many disorders associated with low brain GABA levels.

2. Materials and methods

2.1. Compounds

The compounds used in the present study are detailed in Table 1. GABA, dl-dithiothreitol, bovine serum albumin (BSA), 2-oxoglutarate, 3,5-diaminobenzoic and succinic semialdehyde were acquired from Sigma–Aldrich (Milan, Italy). Reagents were dissolved in MilliQ deionized water. All other materials were from standard local sources and of the highest grade commercially available.

2.2. Animals

All experiments were performed in strict compliance with the recommendation of the EEC (86/609/CEE) for the care and use of laboratory animals and were approved by the Animal Care and Ethics Committee of the University of Siena, Italy.

The study was carried out on adult male New Zealand albino rabbits (Charles River, Calco, Como, Italy), weighing 2.0–2.5 kg and kept in large individual cages, under a 12:12 h day–night cycle at 20 °C ambient temperature. Drinking water and conventional laboratory rabbit food were available ad libitum.

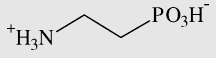
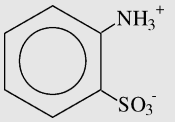
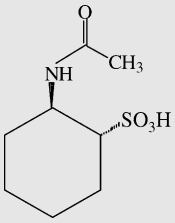
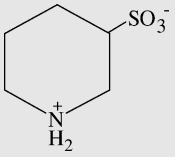
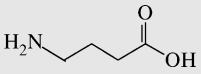
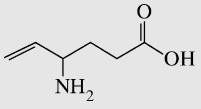
2.3. Enzyme preparation

Rabbit brain GABA-T was partially purified by following the first two steps of the method described by Fowler and John [23], with slight modifications. Enzyme preparation procedures were carried out at 4 °C, unless specified otherwise. A glass Potter–Elvehjem fitted with a teflon pestle was used to prepare a 33% (w/v) whole brain homogenate in buffer solution (pH 7.4) containing sodium acetate (10 mM), EDTA (1 mM), pyridoxal phosphate (0.1 mM), 2-oxoglutarate (1 mM) and 2-mercaptoethanol (0.1 mM). The homogenate was then acidified to pH 5.3 with 10% (v/v) acetic acid and added with an ammonium sulfate solution up to 25% saturation to protect the enzyme from heat. The suspension was placed in a water bath and the temperature brought up to 53 °C for 5 min. After cooling to 4 °C, heat-labile proteins were removed by centrifugation at 5000 *g* for 20 min. Ammonium sulfate was added to the supernatant and the proteins that precipitated between 45 and 65% $(NH_4)_2SO_4$ saturation were separated by centrifugation at 10000 *g* for 30 min and redissolved in 10 mM Tris–HCl containing 10 mM sodium acetate, adjusted to pH 7.5. The solution thus obtained, containing GABA-T, was dialysed overnight against 10 mM HCl, 10 mM sodium acetate and adjusted to pH 7.5 with solid Tris. All the experiments were performed in buffer A (0.1 mM EDTA, 0.5 mM dithiothreitol and 0.1 mM KH_2PO_4) adjusted to pH 8.4 with NaOH.

2.4. Assay of GABA-T activity

Rabbit brain GABA-T-specific activity was assayed by using the fluorimetric method of Salvador and Albers [24]. The transaminase assay was based upon the measurement of succinic semialdehyde produced from GABA during incubation of the enzyme. Briefly, a buffered substrate solution (BSS) was prepared containing 0.1 M α-ketoglutaric acid and 0.25 M γ-aminobutyric acid adjusted to pH 8.4 with NaOH. Aliquots of

Table 1 – Compounds used in the present study

Compound (abbreviation)	Structure	Origin	Inhibition type (K_i)
2-Aminoethane phosphonic acid (AEP)		Sigma–Aldrich	Mixed (1010 μM)
2-Aminobenzene sulfonate (ANSA)		Sigma–Aldrich	Competitive (434 μM)
2-(N-acetylamino) cyclohexane sulfonic acid (ATAHS)		Synthesised [21]	Competitive (598 μM)
(±)Piperidine-3-sulfonic acid (PSA)		Synthesised [22]	Mixed (364 μM)
GABA		Sigma–Aldrich	–
γ-Vinyl GABA		Gift[a]	Competitive (320 μM)

The purity of the compounds synthesised at the department was found to be higher than 95% by ^{1}H NMR or high-performance liquid chromatography (HPLC). K_i represents the inhibition constant value for competitive inhibition. For further details, see Section 2.7.

[a] Vigabatrin (Camillo Corvi S.p.A, Italy) was a kind gift of Dr. M. Ulivelli.

12 μl of BSS were added to 25–400 μg proteins and the final volume adjusted to 250 μl with buffer A. Incubation was carried out in a water bath at 38 °C for 2 h. Afterwards, equal volumes of sample and reagent (0.25 M 3,5-diaminobenzoic acid adjusted to pH 6 with K_2CO_3) were mixed and heated for 1 h in a water bath at 60 °C. A 15 μl aliquot of the reaction mixture was then diluted with 3.0 ml of MilliQ deionized water and the fluorescence of the diluted aliquot was recorded at excitation and emission wavelengths of 405 and 492 nm, respectively (spectrofluorophotometer Shimadzu RF-5000, Tokyo, Japan). A standard curve of SSA concentrations versus fluorescence was obtained for each set of samples. The fluorescence background corresponded to 3.3×10^{-5} M SSA, i.e. one order of magnitude lower than that reported by Salvador and Albers [24]. One unit (U) of enzyme is defined as that which formed 1 nmole of SSA in 1 min. Protein concentration was determined by the method of Bradford [25].

2.5. Determination of time-dependent and irreversible GABA-T inactivation by VIGA and taurine analogues

Partially purified GABA-T was incubated at 37 °C in buffer A containing various concentrations of VIGA (100–400 μM) or TA (each tested at concentrations up to four times their K_i value) and assayed for its activity. The addition of the inhibitors even at the highest concentrations tested, did not modify the pH of the assay mixture.

At various time intervals (0, 2.5, 5, 10, 15 min) aliquots of the enzyme solution were removed, diluted 1:20 with buffer A and assayed for GABA-T activity in the presence of GABA and 2-oxoglutarate at 30 and 12.5 mM final concentration, respectively. Time-dependent inhibition was plotted as linear decrease of log residual enzyme activity versus pre-incubation time. For the assessment of irreversible inhibition, compounds at $3 \times K_i$ concentration were pre-incubated with the partially purified enzyme for 1 h at 37 °C and incubation solutions processed as described above.

2.6. Determination of GABA content in rabbit cortical slices after the incubation with VIGA and taurine analogues

To verify whether VIGA and TA by acting as GABA-T inhibitors could promote GABA accumulation in brain tissues, rabbit brain cortical slices – prepared as previously described [26] – were incubated with the drugs and the amount of GABA determined by using a NMR assay method [27]. Briefly, after the brain excision, cortices were isolated and placed in oxygenated (95% O_2 + 5% CO_2) artificial cerebrospinal fluid (ACSF) with the following composition (mM): 120 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 1.0 NaH_2PO_4, 1.5 $CaCl_2$, 26 $NaHCO_3$, 11 glucose, final pH 7.4. Tissue was then cut into slices with a manual chopper (Stoelting Co.,

Wood Dale, IL, USA) and dipped in ice-cold ACSF. To minimize surface damage and hypoxia due to tissue cutting a 350- to 400-μm thickness of slices was programmed [28,29]. All tissue manipulation was carried out in a refrigerated room at 4 °C.

Slices were incubated for 3 h with VIGA or TA at their K_i concentration in ACSF containing glutamine and NH_4Cl. Ammonium chloride was added to stimulate glial glutamine synthesis that along with the added glutamine would stimulate GABA synthesis [30,31]. Rapid freezing blocked the reaction. Slices were subsequently thawed and extracted in 2 ml of a cold 12% perchloric acid (PCA) solution, containing 7.69 mM dichloracetic acid. After careful mixing and centrifugation at 3200 *g* for 15 min at 4 °C, the supernatant thus obtained was brought to a neutral pH with a solution containing 4.8 M KOH and 0.3 M K_2HPO_4 and again centrifuged at 3200 *g* for 10 min at 4 °C. The final neutral supernatant was mixed with 0.5 g of a chelating resin (Chelex® 100, Sigma–Aldrich, Milano, Italy), filtered and then lyophilized.

The dried powder thus obtained was dissolved in neutral 50 mM deuterated phosphate in D_2O containing 2 mM isopropanol. Dichloracetic acid was used in the extraction procedure as an internal standard and a chemical shift reference.

NMR measurements were carried out at 14.1 T with a Bruker Avance 600 MHz spectrometer operating at controlled temperature (295 ± 0.1 K) and equipped with a Silicon Graphics workstation. A 5 mm Triple Broadband Inverse (TBI) probe was used for ^1H experiments.

2.7. Data analysis

Kinetic parameters (K_m and V_{max}) were calculated according to Michaelis–Menten equation for one or two enzymes by nonlinear regression analysis (Prism 3.03 Graphpad Software Inc., San Diego, CA, USA).

Inhibition constant values for competitive inhibitors (K_i) were provided by the negative *x*-intercept from secondary plots of slopes obtained with Lineweaver–Burk analysis, versus concentration of inhibitor, as described by Dixon and Webb [32]. In the case of mixed inhibitors, the K_i^* (non-competitive inhibition constant) was determined by replotting *y*-intercepts versus inhibitor concentration [32]. Time-dependent inhibition was evaluated on a two-step model of a second-order reaction followed by an irreversible first-order reaction, i.e.:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{inact}} E - I \quad (1)$$

where k_{inact} represents the limiting rate for time-dependent inhibition, which is slow as compared to the catalytic turnover. From this model, the dependence of the observed rate on the inhibitor concentration is defined by

$$k_{obs} = \frac{k_{inact}[I]}{K_I + [I]} \quad (2)$$

where $K_I = (k_{-1}/k_{+1})$ and represents the dissociation constant for inhibitor binding to free enzyme. Eq. (2) can be transformed into the more familiar linearised form by changing the terms of the equation into their reciprocals, i.e.:

$$\frac{1}{k_{obs}} = \frac{K_I}{k_{inact}} \frac{1}{[I]} + \frac{1}{k_{inact}} \quad (3)$$

Values of k_{inact} and K_I were obtained from a linear least-squares fit of $(1/k_{obs})$ versus $1/[I]$, where the reciprocal of the *y*-intercept represents k_{inact} and the negative reciprocal of the *x*-intercept represents K_I [33,34]. All the experiments were performed by using rabbit brain enzyme preparations derived from at least five individual animals. Values obtained are expressed as mean ± S.E.M.; one-way analysis of variance (ANOVA), followed by Dunnett's test for multiple comparisons, was used.

3. Results

3.1. Specific activity, K_m and V_{max} of partially purified rabbit brain GABA-T

Preliminary assays of GABA-T activity showed that the amount of SSA formed increased linearly with time up to 3 h and that the initial rates were proportional to the amount of enzyme preparation proteins added up to 1 mg/ml. The omission of either α-ketoglutarate or GABA in the BSS hampered the formation of ASS. Finally, in partially purified GABA-T preparations, succinic semialdehyde dehydrogenase activity was not detected under the present experimental conditions. Taken together, these observations indicate that the reaction products depend on GABA-T activity.

GABA-T activity measured in crude rabbit brain homogenates was 3.4 ± 0.05 (n = 5) U/mg protein, while in partially purified preparations was 51.2 ± 0.74 (n = 5) U/mg protein, indicating that the enzyme was purified 15-fold (33% yield) in respect to the initial brain homogenate.

The relationship between GABA concentration and GABA-T activity, expressed as formation rate of SSA, followed the Michaelis–Menten equation. The apparent K_m value was 12.8 mM, while maximum reaction velocity (V_{max}) was 6.0 U/mg protein. Rabbit brain GABA-T exhibited only one binding site for the substrate as indicated by the linearity of Eadie Hofstee plot (data not shown).

3.2. Kinetic analysis of the inhibition of GABA-T by TA and VIGA

The mechanism of inhibition of rabbit brain GABA-T by TA and VIGA was determined by measuring enzyme activity at various GABA concentrations (7.5–50 mM) in the presence of increasing concentrations of the inhibitor.

As shown in Fig. 1, panels A and B, PSA and AEP exerted a linear mixed-type inhibition. K_m and V_{max} values, in fact, were both affected by the inhibitor (K_m was increased while V decreased). When plotting the slope or *y*-intercept obtained in the Lineweaver–Burk processing of data versus the inhibitor concentration, a straight line was generated and values for apparent K_i and K_i^* could be obtained (Fig. 1, panels C and D). For both compounds K_i was about three times smaller than the K_i^*, constant for non-competitive inhibition, i.e. 364 and 1154 μM for PSA and 1010 and 2913 μM for AEP, respectively.

On the contrary, ANSA, ATAHS and VIGA behaved like "pure" competitive inhibitors (Fig. 2, panels A and B, and Fig. 3, panel A, respectively) since they increased apparent K_m value while not affecting V_{max}. The respective inhibition constants

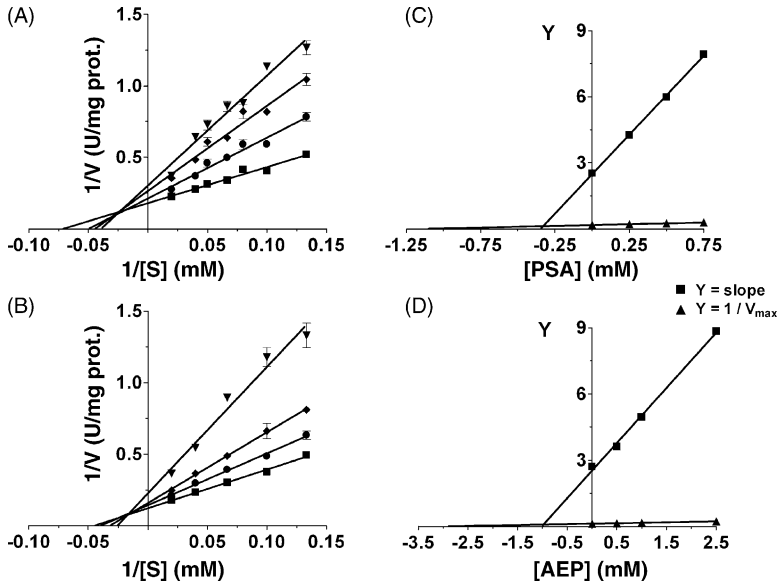


Fig. 1 – Kinetic analysis of partially purified rabbit brain GABA-T in presence of PSA and AEP. Panel A: primary Lineweaver–Burk plot (L/B) of GABA-T activity in absence (■) or in presence of PSA: (●) 250 μM; (◆) 500 μM; (▼) 750 μM. Panel B: L/B plot of GABA-T activity in absence (■) or in presence of AEP: (●) 500 μM; (◆) 1000 μM; (▼) 2500 μM. *S* indicates substrate (GABA) and *V* indicates SSA formation rate expressed as U/mg prot (1 U of enzyme activity was defined as 1 nmole of SSA formed per minute). Panels C and D are secondary plots of the L/B: $1/V_{max}$ (▲) or slope (■) vs. inhibitor concentrations (PSA, panel C or AEP, panel D). Data are reported as mean ± S.E.M. derived from at least five animal preparation. Curves were fitted by a straight-line equation for linear regression and r^2 was 0.99 for each line.

(K_i) are shown in panels C and D of Fig. 2 and panel B of Fig. 3. The most potent GABA-T competitive inhibitor was VIGA (K_i, 320 μM), followed by ANSA and ATAHS (K_i, 434 and 598 μM, respectively).

3.3. *Time-dependent inhibition of GABA-T by VIGA and taurine analogues*

The enzyme inactivation kinetic at various concentrations of VIGA, is presented in Fig. 3, panel C. The dissociation constant for the initial, reversible complex (K_I), calculated by transferring the data into a Kitz–Wilson plot, was 773 μM (Fig. 3, panel D). The rate of formation of the irreversibly inhibited enzyme, k_{inact}, was 0.14 min^{-1} and the efficiency of VIGA with respect to GABA-T, expressed as a function of the k_{inact}/K_I, was 1.81×10^{-4} min^{-1} μM^{-1}. On the contrary, TA did not affect the enzyme activity as a function of pre-incubation time, indicating that the inhibition afforded by them was not time-dependent (data not shown).

3.4. *Reversibility of the inhibition of GABA-T by VIGA and taurine analogues*

Reversibility of the inhibition of GABA-T by VIGA and TA was assessed by determining the recovery of GABA-T activity upon dilution of the enzyme/inhibitor mixture, pre-incubated as previously described. As shown in Fig. 4, after pre-incubating the enzyme with VIGA, the activity was not restored following dilution of the reaction mixture. On the contrary, TA exerted a reversible inhibition since recovery of the activity was observed. In particular, for PSA and ATAHS the restoring of enzyme activity was almost complete, while for AEP and ANSA a partial inhibition was still operant after dilution owing to the fact that the inhibitor was present in the diluted samples at a concentration of about $0.15 \times K_i$, found to inhibit GABA-T by 30%.

3.5. *GABA content in rabbit cortical slices after treatment with VIGA and taurine analogues*

To verify whether TA acting as GABA-T inhibitors and VIGA itself promoted GABA accumulation in brain tissues, rabbit brain cortical slices were incubated with the drugs at the respective K_i concentration and the amount of GABA determined at the end of incubation. Basal GABA content measured in control slices by NMR analysis was 1.22 ± 0.05 nmoles/mg tissue. As shown in Fig. 5, GABA concentration was significantly increased after incubation with VIGA or TA. In particular, with TA, this increase averaged between 32.0 ± 7.8% (ATAHS) and 65.0 ± 13.0% (ANSA) over control values, respectively ($P < 0.01$).

4. Discussion

The well-known GABA-T inhibitor VIGA, indicated for partial- and pharmacoresistant-epilepsy, suffers from several major drawbacks, the most severe of which is the induction of visual field defects in patients [35]. This demands further research in this field to discover novel and safer GABA-T inhibitors.

The aim of the present investigation was to define the mechanism(s) by which some TA previously shown to be

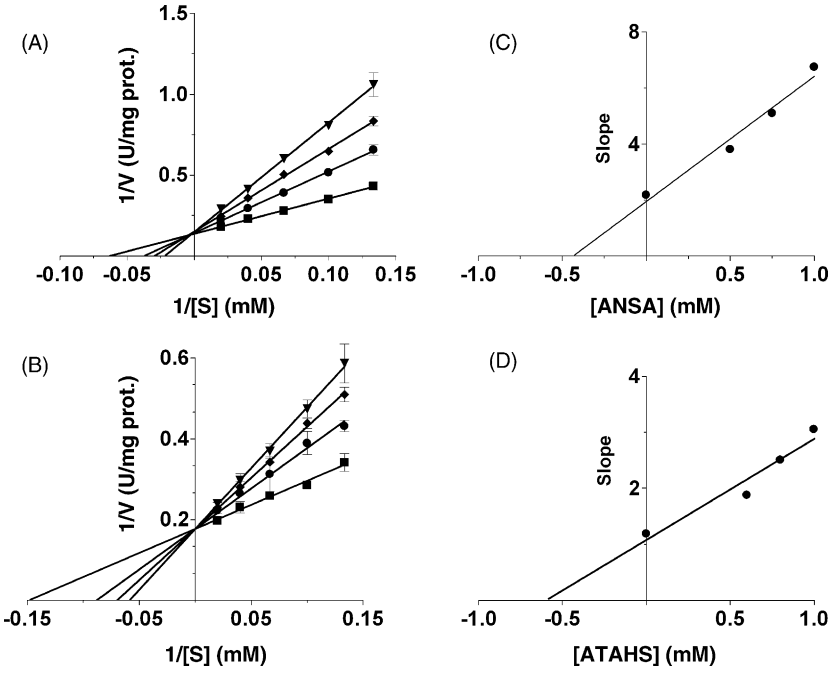


Fig. 2 – Kinetic analysis of partially purified rabbit brain GABA-T in presence of ANSA and ATAHS. Panel A: primary Lineweaver–Burk plot (L/B) of GABA-T activity in absence (■) or in presence of ANSA: (●) 500 μM; (◆) 750 μM; (▼) 1000 μM. Panel B: L/B plot of GABA-T activity in absence (■) or in presence of ATAHS: (●) 600 μM; (◆) 800 μM; (▼) 1000 μM. *S* indicates substrate (GABA) and *V* indicates SSA formation rate expressed as U/mg prot (1 U of enzyme activity was defined as 1 nmole of SSA formed per minute). Panels C and D are secondary plots of the L/B line slopes vs. inhibitor concentrations (ANSA, panel C or ATAHS, panel D). Data are reported as mean ± S.E.M. derived from at least five animal preparations. Curves were fitted by a straight-line equation for linear regression and r^2 ranged between 0.97 and 0.99.

GABA-T inhibitors in rabbit brain homogenates [20] act on rabbit brain partially purified enzyme. PSA, ANSA and ATAHS proved to be the most interesting compounds since they were reversible, not time-dependent inhibitors with K_i values close to that of VIGA (Table 1). Moreover, all the novel GABA-T inhibitors promoted a significant increase of GABA content in rabbit brain slices, which is ascribable to their ability to slow down cytosolic GABA degradation.

PSA and AEP behaved like fully mixed inhibitors since they increased the apparent K_m and decreased *V* of the enzyme. In this case the inhibitor can bind both to the free enzyme and to the enzyme–substrate complex and, as a result, it slows down the breakdown of this complex [34]. This type of inhibition generally results from the combination of a partially competitive and a pure, non-competitive inhibition. However, for both compounds, the competitive inhibition was predominant since K_i was one-third K_i^*. This was also the case of 1-anilinonaphthalene-8-sulfonic acid which is a mixed-type inhibitor towards NADPH:quinone oxidoreductase of ζ-crystallin [36]. Furthermore, the increase of K_m value observed with PSA and AEP indicates that these compounds caused a decrease of the affinity of GABA toward the active site of the enzyme. This suggests that they might induce conformational changes of GABA-T structure; in this case, in fact, both inhibitor and substrate do not exclude each other but both bind to the enzyme, independently. Moreover, PSA and AEP can cause a decrease in enzyme activity by preventing the enzyme–substrate complex from being broken down, thus behaving like partially mixed inhibitors. However, the linearity of the curves obtained by plotting the velocity versus the concentration of the inhibitors (data not shown) rules out this hypothesis.

AEP was the least potent among the compounds studied ($K_i > 1000$ μM) and for this reason cannot be considered of great pharmacological interest as a GABA-T inhibitor. Furthermore, this compound shows an affinity in the micromolar range for rabbit brain $GABA_B$ receptors [20] and in vivo causes a dose-related hyperthermia [37], thus suggesting that it behaves like a $GABA_B$ antagonist in the central mechanisms of thermoregulation. However, since $GABA_B$ antagonists might have excitatory and proconvulsive effects [38], AEP $GABA_B$ antagonistic properties represent an overwhelming problem in the light of a possible use in those pathological conditions with mismatched inhibitory synaptic transmission like in epilepsy. PSA turned out to be the most effective inhibitor of GABA-T (K_i 364 μM). Recently, we have demonstrated that in vivo it induces a dose-related hypothermia accompanied by inhibition of gross motor behaviour [37]. Also VIGA is able to decrease body temperature in many mammals [39–42] by raising brain GABA contents [27]. Coherently, PSA in the present report has been shown to increase significantly the GABA content in rabbit brain slices. On the other hand PSA is not only a GABA-T inhibitor, but it binds also to $GABA_A$ receptors of rabbit [20] and, as originally reported by Krogsgaard-Larsen et al. of rat brains [43]. Its $GABA_A$ affinity is very close to its K_i for GABA-T [20]. Although it does not behave like a pure GABA-T inhibitor, only from a more general

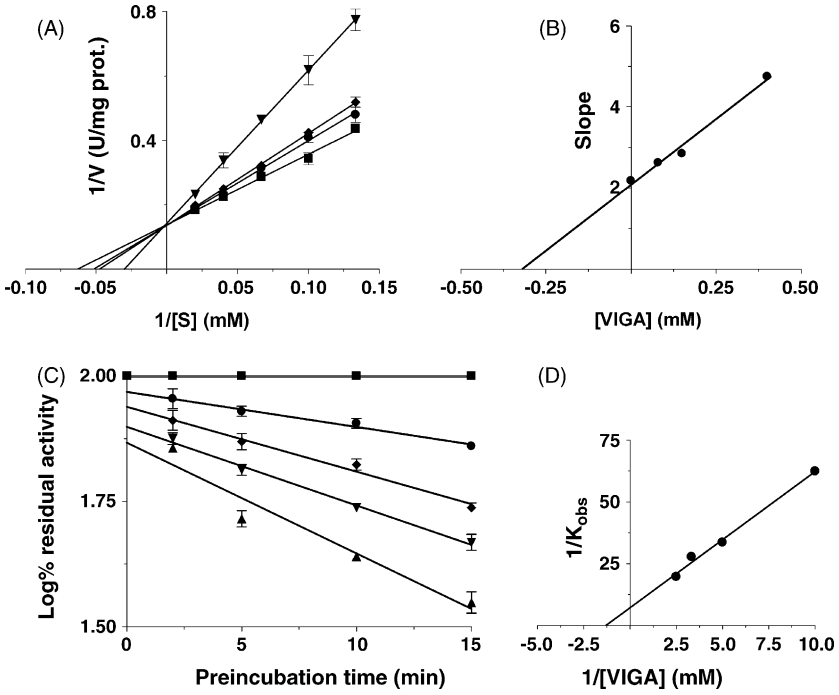


Fig. 3 – Kinetic analysis of partially purified rabbit brain GABA-T in presence of VIGA. Panel A: primary Lineweaver–Burk plot (L/B) of GABA-T activity in absence (■) or in presence of VIGA: (●) 80 μM; (◆) 150 μM; (▼) 400 μM. *S* indicates substrate (GABA) and *V* indicates SSA formation rate expressed as U/mg prot (1 U of enzyme activity was defined as 1 nmole of SSA formed per minute). Panel B: secondary plot from L/B line slopes versus VIGA concentrations. Panel C: Kinetic parameters for the time-dependent inhibition of GABA-T by VIGA. Initial rates were determined after pre-incubation of enzyme in absence (■) or in presence of VIGA: (●) 100 μM; (◆) 200 μM; (▼) 300 μM (▲) 400 μM for various time periods (0–15 min) and expressed as percent of control rate obtained in the absence of any inhibitor. Results are presented graphically in semi-log plots of residual activity as a function of pre-incubation time. Curves were fitted by a straight-line equation for linear regression and r^2 ranged between 0.96 and 0.99. Panel D: secondary plot of $1/K_{obs}$ vs. 1/[VIGA] (mM), according to Kitz and Wilson [33]. The reciprocal of *y*-intercept and the negative reciprocal of *x*-intercept represent k_{inact}, the inactivation rate of enzyme, and K_I, the dissociation constant for the initial reversible complex, respectively.

point of view, being a compound that potentiates GABA-ergic system by acting simultaneously through different mechanisms, it might prove useful. Interestingly, VIGA, besides being a GABA-T inhibitor, is also able to block GABA transporter GAT1 of mouse brain at extremely low (nanomolar range) as well as extremely high (millimolar range) concentrations [44].

PSA, as well as ATAHS and ANSA, due to their different mechanism of GABA-T inhibition in comparison to VIGA, may be considered lead compounds for a new class of GABA-T inhibitors, susceptible of therapeutic exploitation in many disorders associated with low levels of GABA. Their usefulness as specific tools, however, might be hampered by their poor ability to gain access into the central nervous system (CNS). This problem can be solved by conjugating them to compounds that are effectively transported through the blood brain barrier into the brain. Jacob et al. [45] have demonstrated that 1-*O*-linolenoyl-2-*O*-(4-aminobutyryl)-3-*O*-(4-vinyl-4-aminobutyryl)glycerol (LGV) is a pro-drug which readily overcome the blood–brain barrier and releases upon hydrolysis both GABA and VIGA into CNS. Interestingly, in respect to VIGA, LGV in vivo was active at a 300-fold lower dose and it has a more rapid action onset time.

ANSA and ATAHS were shown to be competitive GABA-T inhibitors with potency close to that of VIGA. They behaved like reversible inhibitors, suggesting that they bind to the same enzyme site as the substrate, i.e. at the active centre. Their competitive and reversible properties might be due to the fact that these compounds do not possess such a reactive group like the vinylic moiety of VIGA. This drug, in fact, irreversibly inhibits mammalian brain GABA-T because it is initially accepted as a substrate and acts as a competitive inhibitor, directly interacting with the enzyme active site.

The fine mechanism for its GABA-T inactivation has been investigated in the past with various biochemical techniques and resulted in several proposals (for more details see Storici et al. [46]). Essentially, the irreversibility depends on the covalent binding of the active complex PLP-VIGA to a nucleophilic residue of GABA-T active site. Consequently, VIGA action is long lasting, the synthesis of new enzyme being necessary to overcome that effect, and specific because the drug exploits the enzyme own catalytic mechanism to precipitate its irreversible inhibition. However, GABA-T inhibition by VIGA is hypothesised to be at the basis of its adverse effects. Visual field loss in VIGA-treated epileptic patients is both dose dependent [47,48] and irreversible [49] and results from a sequence of events starting from cone cell injury to a more severe disorganization of the photoreceptor layer [16]. In animals VIGA was found to cross the

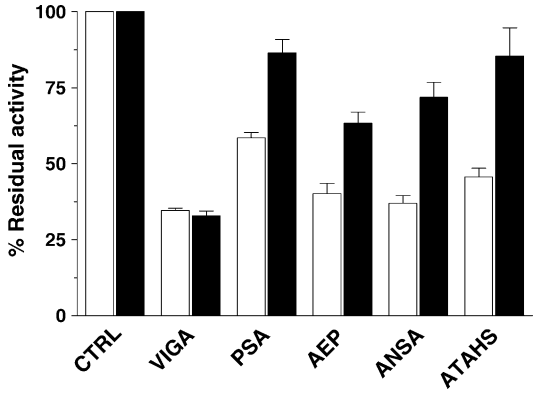


Fig. 4 – Reversibility of partially purified rabbit brain GABA-T inhibition by VIGA and taurine analogues. GABA-T was pre-incubated in the presence of (final concentration) 400 μM VIGA, 1000 μM PSA, 4000 μM AEP, 2000 μM ANSA or 2000 μM ATAHS at 37 °C for 1 h, and then diluted 20 times with buffer A. Details of the experimental procedures were described in Section 2. Data are reported as mean (% over CTRL) ± S.E.M. derived from at least five animal preparations. CTRL bars represent 100% GABA-T activity in the absence of inhibitor. Open bars represent samples containing 50.0 U/ml of enzyme; black bars diluted samples (2.5 U/ml). The enzyme activity was calculated in all samples at the end of incubation period.

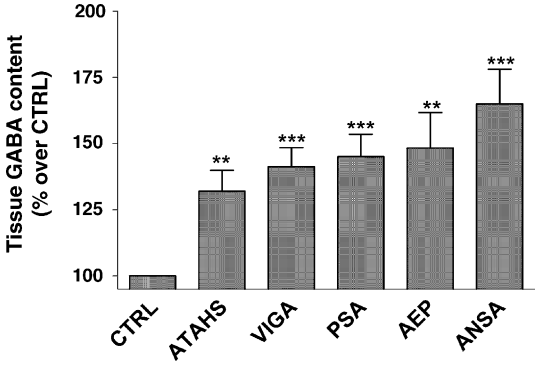


Fig. 5 – GABA content in rabbit cortical slices after treatment with VIGA and taurine analogues. Rabbit brain cortical slices were incubated for 3 h with the drugs at their K_i concentration, as described in Section 2. Basal GABA content measured in non-treated rabbit cortical slices (CTRL) was 1.22 ± 0.05 nmoles/mg tissue. Data are reported as mean (% over CTRL) ± S.E.M. derived from at least five animal preparations and one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons was used. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

blood–retinal barrier and reach in the retina a concentration fivefold higher than that attained in the brain [50], giving rise to an excessive GABA accumulation in the retina [51]. Although the hypothesis that GABA itself is implicated in the VIGA-elicited retinal damage has been advanced [16], a recent report demonstrates that in albino rats acute VIGA exposure damages the outer retina by a GABA-independent and VIGA-specific mechanism, which consists in sensitising photoreceptors (and possibly Müller cells) to light-induced damage [17]. In this context, reactive oxygen species (ROSs) are probably involved since they participate to light-mediated retinal toxicity [52]. Consequently, novel compounds with a GABA-T inhibition mechanism different from that of VIGA are needed. Furthermore, it is worth mentioning that GABA-T inhibitors here investigated are structural analogues of taurine. This amino acid seems to play an important role not only in the physiology of normal vision [53–57] but also in the pathogenesis of retinal degeneration, diabetic retinopathy and macular edema [55,56,58,59]. Recently, taurine has been shown to protect photoreceptor outer segment cell membranes against light-induced oxidative damage [60]. In consideration of the involvement of ROSs in retinal light-mediated toxicity and the neuroprotective role of taurine [61], we envisage a neuroprotective effect by these TA worth to be assessed.

Further advances in the development of more effective anticonvulsant drugs depend upon the elucidation of the structure and mode of action of GABA-T. However, most studies on GABA-T, including the present one, have been carried out on mammalian enzymes other than the human form. Even though studies of the amino acid sequence have revealed that GABA-T is highly conserved along the various mammalian species investigated, the human enzyme has been proven to be immunologically distinct from the other mammalian enzymes [62,63]. However, the K_m value for GABA and the catalytic activity of human recombinant GABA-T are not significantly different from those of other mammalian enzymes such as pig and cow [62–64]. In contrast, the difference was more pronounced in the inactivation by VIGA since bovine GABA-T required a much higher concentration of VIGA for the same level of inactivation, compared to human GABA-T [62]. Interestingly enough, the present results show that the rabbit enzyme is as sensitive to VIGA as the human form, being K_I and k_{inact} values for both enzymes very similar. Therefore, rabbit brain GABA-T seems to be the ideal enzyme for performing preliminary screenings on novel compounds, potential human GABA-T inhibitors.

In conclusion, the present study introduces novel TA, with an inhibition mechanism of GABA-T different from that of VIGA, which might be useful for treating pathological conditions caused by a deficient intracerebral GABA activity.

Acknowledgements

This work was supported by University of Siena, PAR Progetti. The authors thank Dr. M. Ulivelli (Neuroscience Department, Faculty of Medicine, University of Siena) for supplying vigabatrin.

REFERENCES

[1] Baxter CF, Roberts E. The gamma-aminobutyric acid-alpha-ketoglutaric acid transaminase of beef brain. J Biol Chem 1958;233:1135–9.

[2] Mehta PK, Christen P. The molecular evolution of pyridoxal-5′-phosphate-dependent enzymes. Adv Enzymol Relat Areas Mol Biol 2000;74:129–84.
[3] Cooper AJL. Glutamate-gamma-aminobutyrate transaminase. Methods Enzymol 1985;113:80–2.
[4] Bakay RA, Harris AB. Neurotransmitter, receptor and biochemical changes in monkey cortical epileptic foci. Brain Res 1981;206:387–404.
[5] Lloyd KG, Munari C, Worms P, Bossi L, Bancaud J, Talairach J, et al. The role of GABA mediated neurotransmission in convulsive states. Adv Biochem Psychopharmacol 1981;26:199–206.
[6] Butterworth J, Yates CM, Simpson J. Phosphate-activated glutaminase in relation to Huntington's disease and agonal state. J Neurochem 1983;41:440–7.
[7] Nishino N, Fujiwara H, Noguchi-Kuno SA, Tanaka C. $GABA_A$ receptor but not muscarinic receptor density was decreased in the brain of patients with Parkinson's disease. Jpn J Pharmacol 1988;48:331–9.
[8] Aoyagi T, Wada T, Nagai M, Kojima F, Harada S, Takeuchi T, et al. Increased gamma-aminobutyrate aminotransferase activity in brain of patients with Alzheimer's disease. Chem Pharm Bull (Tokyo) 1990;38:1748–9.
[9] Schwartz-Bloom RD, Sah R. Gamma-aminobutyric acid(A) neurotransmission and cerebral ischemia. J Neurochem 2001;77:353–71.
[10] Gunne LM, Haggstrom JE, Sjöquist B. Association with persistent neuroleptic-induced dyskinesia of regional changes in brain GABA synthesis. Nature 1984;309: 347–9.
[11] Silverman RB. Mechanism-based enzyme inactivators. Methods Enzymol 1995;249:240–83.
[12] Nanavati SM, Silverman RB. Design of potential anticonvulsant agents: mechanistic classification of GABA aminotransferase inactivators. J Med Chem 1989;32: 2413–21.
[13] Sarup A, Larsson OM, Schousboe A. GABA transporters and GABA-transaminase as drug targets. Curr Drug Targets CNS Neurol Disord 2003;2:269–77.
[14] Gidal BE, Privitera MD, Sheth RD, Gilman JT. Vigabatr: a novel therapy for seizure disorders. Ann Pharmacother 1999;33:1277–86.
[15] Sills GJ, Butler E, Forrest G, Ratnaraj N, Patsalos PN, Brodie MJ. Vigabatrin, but not gabapentin or topiramate, produces concentration-related effects on enzymes and intermediates of the GABA shunt in rat brain and retina. Epilepsia 2003;44:886–92.
[16] Duboc A, Hanoteau N, Simonutti M, Rudolf G, Nehlig A, Sahel JA, et al. Vigabatrin, the GABA-transaminase inhibitor, damages cone photoreceptors in rats. Ann Neurol 2004;55:695–705.
[17] Izumi Y, Ishikawa M, Benz AM, Izumi M, Zorumski CF, Thio LL. Acute vigabatrin retinotoxicity in albino rats depends on light but not GABA. Epilepsia 2004;45:1043–8.
[18] Wilson EA, Brodie MJ. Chronic refractory epilepsy may have role in causing these unused lesions. BMJ 1997;314:1693.
[19] Baulac M, Nordmann JP, Lanoé Y. Severe visual-field constriction and side-effects of GABA-mimetic antiepileptic agents. Lancet 1998;352:546.
[20] Frosini M, Sesti C, Dragoni S, Valoti M, Palmi M, Dixon HB, et al. Interactions of taurine and structurally related analogues with the GABA-ergic system and taurine binding sites of rabbit brain. Br J Pharmacol 2003;138:1163–71.
[21] Cordero FM, Machetti F, Cacciarini M, De Sarlo F. Amino-sulfonation of alkenes in a three-component reaction. Eur J Org Chem 2002;8:1407–11.
[22] Freifelder M, Wright HB. Hypocholesteremic agents. II. The hydrogenation of some pyridinesulfonic and pyridinealkanesulfonic acids. J Med Chem 1964;53:664–5.
[23] Fowler LJ, John RA. The reaction of ethanolamine *O*-sulphate with 4-aminobutyrate aminotransferase. Biochem J 1981;197:149–54.
[24] Salvador RA, Albers RW. The distribution of glutamic-gamma-aminobutric transaminase in the nervous system of the rhesus monkey. J Biol Chem 1959;234: 922–5.
[25] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[26] Meini A, Benocci A, Frosini M, Sgaragli GP, Garcia JB, Pessina GP, et al. Potentiation of intracellular Ca^{2+} mobilization by hypoxia-induced NO generation in rat brain striatal slices and human astrocytoma U-373 MG cells and its involvement in tissue damage. Eur J Neurosci 2003;17:692–700.
[27] Errante LD, Williamson A, Spencer DD, Petroff OA. Gabapentin and vigabatrin increase GABA in the human neocortical slice. Epilepsy Res 2002;49:203–10.
[28] Andrew RD, Mac Vicar BA. Imaging cell volume changes and neuronal excitation in the hippocampal slice. Neuroscience 1994;62:371–83.
[29] Forsythe ID, Tsujimoto T, Barnes-Davies M, Cuttle MF, Takahashi T. Inactivation of presynaptic calcium current contributes to synaptic depression at a fast central synapse. Neuron 1998;20:797–807.
[30] Kapetanovic IM, Yonekawa WD, Kupferberg HJ. Time-related loss of glutamine from hippocampal slices and concomitant changes in neurotransmitter amino acids. J Neurochem 1993;61:865–72.
[31] Martin DL, Tobin AJ. Mechanisms controlling GABA synthesis and degradation in the brain. In: Martom DL, Olsen RW, editors. GABA in the nervous system: the view at fifty years. Philadelphia: Lippincott Williams and Wilkins; 2000. p. 25–41.
[32] Dixon M, Webb EC. Enzyme inhibition and activation. In: Dixon M, Webb EC, editors. Enzymes. London: Longman; 1979. p. 332–49.
[33] Kitz R, Wilson IB. Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. J Biol Chem 1962;237:3245–9.
[34] Tipton KF. Enzymes: irreversible inhibition. In: Nature encyclopedia of life sciences. London: Nature Publishing Group; 2001 (http://www.els.net/), doi:10.1038/npg.els.0000601.
[35] Kalviainen R, Nousiainen I. Visual field defects with vigabatrin: epidemiology and therapeutic implications. CNS Drugs 2001;15:217–30.
[36] Bazzi MD, Rabbani N, Duhaiman AS. Hydrophobicity of the NADPH binding domain of camel lens zeta-crystallin. Biochim Biophys Acta 2001;1546:71–8.
[37] Frosini M, Ricci L, Saponara S, Palmi M, Valoti M, Sgaragli GP. GABA-mediated effects of some taurine derivatives injected i.c.v. on rabbit rectal temperature and gross motor behavior. Amino Acids 2006;30.
[38] Kantrowitz JT, Francis NN, Salah A, Perkins KL. Synaptic depolarizing GABA Response in adults is excitatory and proconvulsive when $GABA_B$ receptors are blocked. J Neurophysiol 2005;93:2656–67.
[39] Schechter PJ, Tranier Y. Effect of GABA-transaminase inhibition on rectal temperature in mice. Pharmacol Biochem Behav 1977;6:667–9.
[40] Shuaib A, Ijaz S, Hasan S, Kalra J. Gamma-vinyl GABA prevents hippocampal and substantia nigra reticulata damage in repetitive transient forebrain ischemia. Brain Res 1992;590:13–7.
[41] Ginefri-Gayet M, Gayet J. Hypothermia induced by infusion of methionine sulfoximine into the dorsal raphe nucleus of

the rat: involvement of 5-HT_{1A} and $GABA_B$ receptors. Eur J Pharmacol 1993;235:189–96.
[42] Wallace AE, Kline AE, Montanez S, Hernandez TD. Impact of the novel anti-convulsant vigabatrin on functional recovery following brain lesion. Restor Neurol Neurosci 1999;14:35–45.
[43] Krogsgaard-Larsen P, Falch E, Schousboe A, Curtis DR, Lodge D. Piperidine-4-sulphonic acid, a new specific GABA agonist. J Neurochem 1980;34:756–9.
[44] Eckstein-Ludwig U, Fei J, Schwarz W. Inhibition of uptake, steady-state currents, and transient charge movements generated by the neuronal GABA transporter by various anticonvulsant drugs. Br J Pharmacol 1999;128:92–102.
[45] Jacob JN, Hesse GW, Shashoua VE. Synthesis, brain uptake, and pharmacological properties of a glyceryl lipid containing GABA and the GABA-T inhibitor gamma-vinyl-GABA. J Med Chem 1990;33:733–6.
[46] Storici P, De Biase D, Bossa F, Bruno S, Mozzarelli A, Peneff C, et al. Structures of gamma-aminobutyric acid (GABA) aminotransferase, a pyridoxal 5′-phosphate, and [2Fe-2S] cluster-containing enzyme, complexed with gamma-ethynyl-GABA and with the antiepilepsy drug vigabatrin. J Biol Chem 2004;279:363–73.
[47] Hardus P, Verduin WM, Engelsman M, Edelbroek PM, Segers JP, Berendschot TT, et al. Visual field loss associated with vigabatrin: quantification and relation to dosage. Epilepsia 2001;42:262–7.
[48] Toggweiler S, Wieser HG. Concentric visual field restriction under vigabatrin therapy: extent depends on the duration of drug intake. Seizure 2001;10:420–3.
[49] Hardus P, Engelsman M, van Veelen CW, Stilma JS. Vigabatrin: balancing effectiveness vs. (irreversible) visual field loss as a side effect. Ned Tijdschr Geneeskd 2000;144:2066–9.
[50] Sills GJ, Patsalos PN, Butler E, Forrest G, Ratnaraj N, Brodie MJ. Visual field constriction: accumulation of vigabatrin but not tiagabine in the retina. Neurology 2001;57:196–200.
[51] Neal MJ, Cunningham JR, Shah MA, Yazulla S. Immunocytochemical evidence that vigabatrin in rats causes GABA accumulation in glial cells of the retina. Neurosci Lett 1989;98:29–32.
[52] Roberts JE. Ocular phototoxicity. J Photochem Photobiol 2001;64:136–43.
[53] Hayes KC, Carey RE, Schmidt SY. Retinal degeneration associated with taurine deficiency in the cat. Science 1975;188:949–51.
[54] Wright CE, Tallan HH, Lin YY, Gaull GE. Taurine: biological update. Annu Rev Biochem 1986;55:427–53.
[55] Bridges CC, Ola MS, Prasad PD, El-Sherbeny A, Ganapathy V, Smith SB. Regulation of taurine transporter expression by NO in cultured human retinal pigment epithelial cells. Am J Physiol Cell Physiol 2001;281:C1825–36.
[56] El-Sherbeny A, Naggar H, Miyauchi S, Ola MS, Maddox DM, Martin PM, et al. Osmoregulation of taurine transporter function and expression in retinal pigment epithelial, ganglion, and muller cells. Invest Ophthalmol Vis Sci 2004;45:694–701.
[57] Rascher K, Servos G, Berthold G, Hartwig HG, Warskulat U, Heller-Stilb B, et al. Light deprivation slows but does not prevent the loss of photoreceptors in taurine transporter knockout mice. Vis Res 2004;44:2091–100.
[58] Geggel HS, Ament ME, Heckenlively JR, Martin DA, Kopple JD. Nutritional requirement for taurine in patients receiving long-term parenteral nutrition. N Engl J Med 1985;312:142–6.
[59] Heller-Stilb B, van Roeyen C, Rascher K, Hartwig HG, Huth A, Seeliger MW, et al. Disruption of the taurine transporter gene (taut) leads to retinal degeneration in mice. FASEB J 2002;16:231–3.
[60] Hillenkamp J, Hussain AA, Jackson TL, Cunningham JR, Marshall J. Effect of taurine and apical potassium concentration on electrophysiologic parameters of bovine retinal pigment epithelium. Exp Eye Res 2006;82:258–64.
[61] Pasantes-Morales H, Cruz C. Taurine and hypotaurine inhibit light induced lipid peroxidation and protect rod outer segment structure. Brain Res 1985;330:154–7.
[62] Jeon SG, Bahn JH, Jang JS, Park J, Kwon OS, Cho SW, et al. Human brain GABA transaminase tissue distribution and molecular expression. Eur J Biochem 2000;267:5601–7.
[63] Jeon SG, Bahn JH, Jang JS, Jang SH, Lee BR, Lee KS, et al. Molecular cloning and functional expression of bovine brain GABA transaminase. Mol Cell 2001;12:91–6.
[64] Qiu J, Pingsterhaus JM, Silverman RB. Inhibition and substrate activity of conformationally rigid vigabatrin analogues with gamma-aminobutyric acid aminotransferase. J Med Chem 1999;42:4725–8.