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Alpidem analogues containing a GABA or glycine moiety as new anticonvulsant agents

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Abstract

Alpidem analogues containing a GABA (**1**–**3**) or glycine (**4**–**6**) moiety were synthesized and their interaction with the GABA/benzodiazepine receptor complex at central (CBR) and peripheral (PBR) level was evaluated. In particular, their ability to modulate the specific binding of [^{3}H]-GABA to washed membrane preparations from the rat cerebral cortex, as well as their effects on human recombinant $GABA_A$ receptors in Xenopus laevis oocytes, were assessed. Results from these in vitro assays showed that the most active compounds were **1** and **4**. Intraperitoneal administration of compound **1** at a dose of 150 mg/kg significantly antagonized pentylenetetrazole-induced seizures in rats and the protective effects were evident for 90 min. However, compound **4** failed to interact with strychnine-sensitive Gly-binding sites. Consistent with these binding results, intraperitoneal administration of compound **4** at 150 mg/kg showed no effect against convulsions induced by strychnine, except for a prolonged time of the latency of convulsions. The results obtained suggest that compound **1** possesses interesting anticonvulsant activity and deserves further investigation as a novel lipophilic GABA derivative.

Keywords: Blood–brain barrier; Inhibitory neurotransmitters; Imidazopyridine compounds

1. Introduction

γ-Aminobutyric acid (GABA) and glycine (Gly) are the main inhibitory neurotransmitters in the mammalian central nervous system (CNS). Both Gly and GABA receptors are coupled to membrane chloride channels, which have very similar multiconductance properties (Krogsgaard-Larsen, 1990). There is electrophysiological evidence that GABA and Gly may share the same chloride conductance channel (Krogsgaard-Larsen, 1990). Gly, however, exhibits unique properties, being an inhibitory neurotransmitter and, simultaneously, a co-agonist of the excitatory neurotransmitter glutamate (Aprison, 1990). The inhibitory effect of Gly is mediated through the strychnine-sensitive Gly-receptor, a ligand-gated ion channel present mainly in the brain and spinal cord. On the other hand, interaction with the strychnine-insensitive Gly binding sites of the *N*-methyl-D-aspartate (NMDA) receptor complex potentiates the excitatory effects of the neurotransmitter glutamate (Aprison, 1990). A decrease in GABA-ergic and Gly-ergic neurotransmissions appears to be involved in the etiology of several neurological disorders, including anxiety, pain and epilepsy. Several efforts have been devoted to discovering and developing drugs capable of modifying the GABA-ergic and Gly-ergic systems because of their potential for treating these pathological states. For the management of epilepsy, in particular, it is well known that currently available drugs often do not provide adequate control of seizures. Therefore, because about 20% of all patients remain refractory to current drugs or suffer from several side effects, there is still a need for new antiepileptic drugs (Lloyd and Morselli, 1983; Krogsgaard-Larsen, 1988; McNamara et al., 1993).

Penetration of the blood–brain barrier (BBB) by GABA and Gly is poor, due to their hydrophilic nature and the absence of active transport systems (Krogsgaard-Larsen,

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1990; Geurts et al., 1998). Administration of large doses of these neurotransmitters are necessary to produce neuropharmacological effects (Toth et al., 1983).

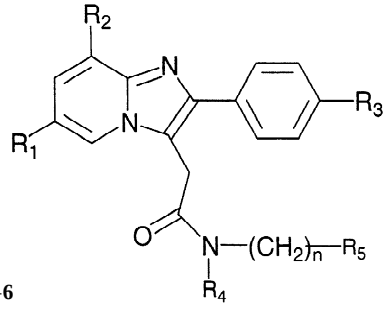
Among the approaches aimed at enhancing the delivery of biologically active substances to the brain, chemical modification of the active compounds, yielding appropriate prodrugs, is widely applied. Therefore, it may be useful to manipulate the molecules of GABA and Gly to obtain compounds with increased lipophilicity, and thereby allow them to gain access to the CNS. In this paper, we describe the synthesis and properties of compounds **1**–**3** and **4**–**6** (Table 1) which are characterized by a GABA- or Gly-ethyl ester moiety linked by an amide bond to an appropriate phenylimidazopyridine portion. This moiety was chosen for two reasons. The first is of a pharmacodynamic nature and is based on the high affinity and selectivity for the GABA-benzodiazepine receptor complex shown by most phenylimidazopyridine compounds, as exemplified by alpidem and zolpidem (Benavides et al., 1993; Durand et al., 1992). The second reason is that the lipophilic phenylimidazopyridine moiety could serve as a carrier for the amino acids taking into account of the high BBB crossing ability shown by most phenylimidazopyridine derivatives (e.g. log C_{brain}/C_{blood} of alpidem=0.4, Durand et al., 1992). We therefore attempted to demonstrate the feasibility of utilizing the covalent attachment of inhibitory neurotransmitter molecules to phenylimidazopyridine portions as carrier groups in order to increase BBB penetration and delivery of the resulting molecules to the target site. A number of advantages were expected: good lipophilicity, BBB penetration, recognition of and delivery enhancement at the target site. Synthesis, binding data and results of electrophysiological studies on compounds **1**–**6**, as well as the anticonvulsant potency of the most active ones, are herein reported and discussed.

2. Materials and methods

2.1. Chemistry

Melting points were determined in open capillary tubes with a Büchi apparatus and are uncorrected. IR spectra were obtained on a Perkin-Elmer 283 spectrophotometer (KBr pellets for solid or nujol for liquid). ^{1}H-NMR spectra were determined on a Varian 390 or Bruker 300 MHz instrument. Chemical shifts are given in δ values downfield from Me_4Si as internal standard. Mass spectra were recorded on a Hewlett-Packard 5995c GC–MS low-resolution spectrometer. All compounds showed appropriate IR, ^{1}H-NMR and mass spectra. Elemental analyses were carried out with a Carlo Erba model 1106 analyzer and results were within ±0.40% of the theoretical values. Silica gel 60 (Merck 70–230 mesh) was used for column

Table 1



Structure, lipophilicity, penetration of BBB of compounds **1**–**6**

Compound	R_1	R_2	R_3	R_4	R_5	n	CLog P[a]	RP-HPLC log k'[b]	PSA[c] (Å^2)	Log BB[d]
1	Cl	H	Cl	H	COOEt	3	4.32	0.74	61.2	−0.11
2	Cl	Cl	H	H	COOEt	3	4.32	0.64	61.7	−0.12
3	Cl	Cl	Cl	H	COOEt	3	5.03	0.86	61.8	−0.01
4	Cl	H	Cl	H	COOEt	1	3.73	0.56	55.4	−0.11
5	Cl	Cl	H	H	COOEt	1	3.74	0.55	55.5	−0.11
6	Cl	Cl	Cl	H	COOEt	1	4.45	0.79	55.4	−0.01
Alpidem	Cl	H	Cl	C_3H_7	C_3H_7	0	5.37			0.40[e]
GABA							−2.77			
Glycine							−3.21			

[a] Calculated according to CLOGP software.
[b] Capacity factors (k') of each solute calculated as: $k'=(t_R-t_0)/t_0$, where t_R is the retention time of the solute and t_0 is the column dead time.
[c] Calculated according to MAREA software.
[d] Calculated according to Clark's model.
[e] Durand et al., 1992.

chromatography. All the following reactions were performed under a nitrogen atmosphere. The starting 2-aminopyridine compounds are commercially available. The preparation of compounds **9** and **10** has previously been reported (Trapani et al., 1999).

2.1.1. General procedure for preparation of N-(3-ethoxycarbonylpropyl) (2-phenylimidazo[1,2-a]pyridine3-yl)-acetamides ***1–3***

To an ice cooled and stirred solution of the suitable ester **10** (1 g) in EtOH (15 ml), 1 M NaOH (3 ml) was added dropwise. After the addition was completed, stirring was prolonged at room temperature for 4 h. Evaporation of the solvent under reduced pressure gave a residue which was dissolved in water, acidified with dilute HCl (pH 1) and the corresponding crude acid **11** was extracted with chloroform (3×30 ml) (90 yield%). To a solution of the crude acid **11** (1.6 mmol) in dry THF (20 ml), CDI (1.8 mmol) was added portionwise and stirred at room temperature for after 15 min; then GABA ethyl ester·HCl (2.4 mmol) was added and stirring prolonged for 30 min. Evaporation of the solvent under reduced pressure gave a residue which was washed with water, extracted with ethyl ether and evaporated. The crude product was purified by silica gel column chromatography [light petroleum ether–ethyl acetate (1:1, v/v) as eluent] to give the required amide. Yields and physical properties of compounds **1–3** are summarized in Table 1.

N-(3-Ethoxycarbonylpropyl) [2-(4-chlorophenyl)-6-chloro-imidazo[1,2-*a*]pyridine3-yl)]-acetamide (**1**): m.p. 178–180 °C. Yield 45%. IR (KBr) 3280, 1720, 1640 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 1.19 (t, J=6 Hz, 3H, CH_3), 1.7–1.8 (m, 2H, CH_2), 2.27 (t, J=6Hz, 2H, CH_2), 3.2–3.3 (m, 2H, CH_2), 3.92 (s, 2H, CH_2), 4.03 (q, J=6Hz, 2H, CH_2), 6.22 (br s, 1H, NH), 7.1–7.6 (m, 6H, Ar), 8.10 (s, 1H, Ar); MS m/z 433 (M^+, 6), 275 (base). Anal. ($C_{21}H_{21}Cl_2N_3O_3$) C, H, N.

N-(3-Ethoxycarbonylpropyl) (2-phenyl-6,8-dichloro-imidazo[1,2-*a*]pyridine3-yl)-acetamide (**2**): m.p. 129–132 °C. Yield 17%. IR (KBr) 3280, 1730, 1630 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 1.18 (t, J=6Hz, 3H, CH_3), 1.7–1.8 (m, 2H, CH_2), 2.25 (t, J=6Hz, 2H, CH_2), 3.2–3.3 (m, 2H, CH_2), 3.87 (s, 2H, CH_2), 4.01 (q, J=6 Hz, 2H, CH_2), 6.53 (br s, 1H, NH), 7.2–7.6 (m, 6H, Ar), 8.09 (s, 1H, Ar); MS m/z 433 (M^+, 11), 275 (base). Anal. ($C_{21}H_{21}Cl_2N_3O_3$) C, H, N.

N-(3-Ethoxycarbonylpropyl) [2-(4-chlorophenyl)-6,8-dichloroimidazo[1,2-*a*]pyridine3-yl)]-acetamide (**3**): m.p. 145–149 °C. Yield 45%. IR (KBr) 3280, 1720, 1635 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 1.19 (t, J=6Hz, 3H, CH_3), 1.7–1.8 (m, 2H, CH_2), 2.28 (t, J=6Hz, 2H, CH_2), 3.2–3.3 (m, 2H, CH_2), 3.81 (s, 2H, CH_2), 4.02 (q, J=6 Hz, 2H, CH_2), 6.83 (br s, 1H, NH), 7.2–7.6 (m, 6H, Ar), 8.05 (s, 1H, Ar); MS m/z 467 (M^+, 6), 311 (base). Anal. ($C_{21}H_{20}Cl_3N_3O_3$) C, H, N.

2.1.2. General procedure for the preparation of N-(ethoxycarbonyl-methyl) (2-phenylimidazo[1,2-a]pyridine3-yl)-acetamides ***4–6***

These compounds were prepared as above by using Gly ethyl ester·HCl instead of GABA ethyl ester·HCl.

N-(Ethoxycarbonyl-methyl) [2-(4-chlorophenyl)-6-chloro-imidazo[1,2-*a*]pyridine3-yl)]-acetamide (**4**): m.p. 177–181 °C. Yield 42%. IR (KBr) 3260, 1740, 1670, 1640 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 1.27 (t, J=6Hz, 3H, CH_3), 3.93 (s, 2H, CH_2), 4.08 (d, J=6 Hz, 2H, CH_2), 4.21 (q, J=6 Hz, 2H, CH_2), 6.95 (br s, 1H, NH), 7.1–7.5 (m, 6H, Ar), 8.06 (s, 1H, Ar); MS m/z 405 (M^+, 8), 275 (base). Anal. ($C_{19}H_{17}Cl_2N_3O_3$) C, H, N.

N-(Ethoxycarbonyl-methyl) (2-phenyl-6,8-dichloro-imidazo[1,2-*a*]pyridine3-yl)-acetamide (**5**): m.p. 119 °C dec. Yield 15%. IR (KBr) 3260, 1740, 1670, 1640 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 1.24 (t, J=6Hz, 3H, CH_3), 3.99 (s, 2H, CH_2), 4.02 (d, J=6 Hz, 2H, CH_2), 4.18 (q, J=6 Hz, 2H, CH_2), 6.13 (br s, 1H, NH), 7.2–7.7 (m, 6H, Ar), 8.11 (s, 1H, Ar); MS m/z 405 (M^+, 15), 275 (base). Anal. ($C_{19}H_{17}Cl_2N_3O_3$) C, H, N.

N-(Ethoxycarbonyl-methyl) [2-(4-chlorophenyl)-6,8-dichloro-imidazo[1,2-*a*]pyridine3-yl)]-acetamide (**6**): m.p. 190–193 °C. Yield 16%. IR(KBr) 1720, 1620 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ: 1.25 (t, J=6Hz, 3H, CH_3), 3.96 (s, 2H, CH_2), 4.02 (d, J=6 Hz, 2H, CH_2), 4.19 (q, J=6 Hz, 2H, CH_2), 6.22 (br s, 1H, NH), 7.2–7.7 (m, 6H, Ar), 8.09 (s, 1H, Ar); MS m/z 439 (M^+, 14), 311 (base). Anal. ($C_{19}H_{16}Cl_3N_3O_3$) C, H, N.

2.1.3. Computational calculations of physicochemical and permeability characteristics of compounds ***1–6***

Lipophilicity and log C_{brain}/C_{blood} (log BB) values were estimated by using CLOGP (v. 4, BioByte) and MAREA (v. 1.4 Department of Pharmacy, Uppsala, Sweden; Stenberg et al., 1999) softwares. The lipophilicity indexes of compounds **1–6**, expressed as log k'=log $(t_R - t_0)/t_0$, were obtained eluting these compounds on a reversed- phase Symmetry C_{18} (25 cm×3.9 mm; 5 μm particles) with methanol–water (75:25, v/v) and a flux of 0.8 ml/min. High-performance liquid chromatography (HPLC) analyses were performed with a Water Associates Model 600 pump equipped with a Water 990 variable wavelength UV detector. The column effluent was monitored continuously at 254 nm. The HPLC mobile phase was prepared from HPLC-grade methanol.

2.2. Biology

Adult male or female Sprague–Dawley CD rats (Charles River, Como, Italy) with body masses of 200–250 g at the beginning the experiments, were maintained under an artificial 12-h light–dark cycle (lights on 08.00 to 20.00 h) at a constant temperature of 23±2 °C and 65% humidity. Food and water were freely available, and the animals

were acclimatized for >7 days before use. Experiments were performed between 08.00 and 14.00 h. Animal care and handling throughout the experimental procedure were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The experimental protocol was approved by the Animal Ethical Committee of the University of Cagliari.

2.3. In vitro receptor binding assays

2.3.1. [^{3}H]-Flunitrazepam binding

The cerebral cortex was homogenized with a Polytron PT 10 in 50 volumes of ice-cold 50 mM Tris–HCl buffer (pH 7.4) and centrifuged twice at 20 000 *g* for 10 min. The pellet was reconstituted in 50 volumes of Tris–HCl buffer and was used for the binding assay. Aliquots of 400 μl of tissue homogenate (0.4–0.5 mg of protein) were incubated in presence of [^{3}H]-flunitrazepam (spec. act. 7.4 Ci/mmol, New England Nuclear) at a final concentration of 0.5 nM, in a total incubation volume of 1000 μl. Drugs were dissolved in dimethylsulfoxide (DMSO) and serial dilutions were made up in DMSO and added in 100 μl aliquots. After a 60-min incubation at 0 °C, the assay was determined by rapid filtration through glass-fiber filter strips (Whatman GF/B). The filters were rinsed with 2- to 4-ml portions of ice-cold Tris–HCl buffer as described above. Radioactivity bound to the filters was quantitated by liquid scintillation spectrometry (Ultima Gold, Camberra Packard). Nonspecific binding was determined as binding in the presence of 5 μM diazepam, and represented about 10% of total binding.

2.3.2. [^{3}H]-PK 11195 binding

After sacrifice, brain and ovary were rapidly removed, the cerebral cortex was dissected and both tissues were stored at −80 °C until assay. The tissues were thawed and homogenized in 50 volumes of Dulbecco's phosphate buffered saline (PBS) pH 7.4 at 4 °C with a Polytron PT 10 (setting 5, for 20 s). The homogenate was centrifuged at 40 000 *g* for 30 min, and the pellet was resuspended in 50 volumes of PBS and recentrifugated. The new pellet was resuspended in 20 volumes of PBS and used for the assay. [^{3}H]-PK 11195 binding was determined in a final volume of 1000 μl tissue homogenate (0.15–0.20 mg protein), 100 μl of [^{3}H]-PK 11195 (spec. act. 85.5 Ci/mmol, New England Nuclear) at final assay concentration of 1 nM, 5 μl of drug solution or solvent and 795 μl of PBS buffer (pH 7.4 at 25 °C). Drugs were dissolved in DMSO and serial dilutions were made up in DMSO and added in 5-μl aliquots. Incubations (0 °C) were initiated by addition of membranes and were terminated 90 min later by rapid filtration through glass-fiber filter strips (Whatman GF/B), which were rinsed with two 4 ml of ice-cold PBS buffer using Cell Harvaster filtration manifold (Brandel). Filter bound radioactivity was quantified by liquid scintillation spectrometry (Ultima Gold, Camberra Packard). Nonspecific binding was defined as binding in the presence of 10 μM unlabelled PK 11195 (Sigma).

2.3.3. [^{3}H]-GABA binding assay

Fresh cerebral cortices were homogenized with a Polytron PT 10 (setting 5 for 30 s) in 10 volumes of ice-cold water and centrifuged 10 min at 48 000 *g* at 0 °C. The pellet was washed once by resuspension and recentrifugation in 10 volumes of 20 mM K_2HPO_4–KH_2PO_4 buffer (pH 7.4) containing 50 mM KCl. The membranes were stored at −20 °C until used 1–15 days later. On the day of the assay the membranes were thawed and centrifuged. The pellet was washed three additional times by resuspension and recentrifugation in ice-cold buffer. The tissue was resuspended in 50 volumes of the same buffer and 700 μl of membrane suspension (500–600 μg of protein) was added to plastic minivials. Drugs were dissolved in DMSO and serial dilutions were made up in DMSO and added in 5-μl aliquots. The total incubation volume was 1 ml. Nonspecific binding was defined as binding in the presence of 1 mM GABA. The incubation (10 min at 4 °C) was started by the addition of 40 nM [^{3}H]-GABA (spec. act. 89 Ci/mmol) and was stopped by centrifugation of the incubation mixture at 48 000 *g* for 10 min. The supernatant was discarded and the pellet was washed gently twice with 4 ml of ice-cold distilled water and was then dissolved in 3 ml of scintillation fluid (Ultima Gold, Camberra Packard).

2.3.4. [^{3}H]-Strychnine binding assay

Rats were killed by guillotine and the spinal cord was rapidly dissected and homogenized on ice with PTFE pestle and a glass homogenizer in 10 volumes of 0.32 M saccharose. The homogenate was centrifuged at 1000 *g* for 10 min at 4 °C and the supernatant was collected and centrifuged at 18 000 *g* for 20 min at 4 °C. The resulting pellet was resuspended by homogenization with a Polytron PT 10 (setting 3 for 30 s) in five volumes of ice-cold distilled water and centrifuged at 8000 *g* for 20 min at 4 °C. The whole supernatant was collected and centrifuged at 40 000 *g* for 20 min at 4 °C. The final pellet was then reconstituted in 1 ml of an ice-cold solution containing (in mM): 4-(2-hydroxyethyl)-piperazine-1-ethanesulphonic acid (HEPES)–Tris, 10; $CaCl_2$, 1; NaCl, 145; KCl, 5; $MgCl_2$, 2.13; glucose, 10 (pH 7.5) and stored at −80 °C until use (1–15 days later). On the day of the assay, the membranes were thawed and reconstituted with a Polytron PT 10 (setting 3 for 30 s) in buffer solution to a final protein concentration of ≈1 mg/ml and used for the binding assay. Protein concentration was determined by the method of Lowry et al. (1951).

[^{3}H]-Strychnine binding was studied by the procedure described by Pfeiffer and Betz (1981) with minor modifications. [^{3}H]-strychnine binding was determined in a final volume of 500 μl consisting of 200 μl of membrane suspension, 50 μl of 8 nM [^{3}H]-strychnine (specific

activity 23 Ci/mol, New England Nuclear), 50 μl of drug solution or solvent, and buffer solution (pH 7.5) to volume. Drugs were dissolved in DMSO and serial dilutions were made up in DMSO and added in 5-μl aliquots. Nonspecific binding was defined as binding in the presence of 100 μM strychnine. Incubations (0 °C) were initiated by addition of membranes and terminated after 60 min by rapid filtration through glass-fiber strips (Whatman GF/B). The filters were rinsed with two 4-ml portions of ice-cold buffer in a Cell Harvester filtration manifold (Model M-24; Brandel Instruments, Gaithersburg, MD, USA), and filter-bound radioactivity was quantified by scintillation spectrometry (Ultima Gold, Camberra Packard).

2.3.5. Functional in vitro studies: electrophysiological studies using Xenopus oocytes

Complementary DNAs encoding the human α1, β2, and γ2L $GABA_A$ receptor subunits were subcloned into the pCDM8 expression vector (Invitrogen, San Diego, CA, USA). The cDNAs were purified with the Promega Wizard Plus Miniprep DNA Purification System (Madison, WI, USA) and then resuspended in sterile distilled water, divided into portions, and stored at −20 °C until used for injection. Stages V and VI oocytes were manually isolated from sections of *Xenopus laevis* ovary, placed in modified Barth's saline (MBS) containing 88 mM NaCl, 1 mM KCl, 10 mM Hepes–NaOH (pH 7.5), 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 0.91 mM $CaCl_2$, and 0.33 mM $Ca(N0_3)_2$ and treated with 0.5 mg/ml of collagenase Type IA (Sigma) in collagenase buffer (83 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes–NaOH, pH 7.5) for 10 min at room temperature, to remove the follicular layer. A mixture of $GABA_A$ receptor α1, β2 and γ2 subunit cDNAs (1.5 ng/30 nl) were injected into the oocyte nucleus using a 10-μl glass micropipette (10–15 μm tip diameter). The injected oocytes were cultured at 19 °C in sterile MBS supplemented with streptomycin (10 mg/ml), penicillin (10 U/ml), gentamicin (50 μg/ml),0.5 mM theophylline, and 2 mM sodium pyruvate. Electrophysiological recordings began approximately 24 h following cDNA injection. Oocytes were placed in a 100-μl rectangular chamber and continuously perfused with MBS solution at a flow-rate of 2 ml/min at room temperature. The animal pole of oocytes was impaled with two glass electrodes (0.5–3 MΩ) filled with filtered 3 M KCl and the voltage was clamped at −70 mV with an Axoclamp 2-B amplifier (Axon Instruments, Burlingame, CA, USA). Currents were continuously recorded on a strip-chart recorder. Resting membrane potential usually varied between −30 and −50 mV. Drugs were dissolved in DMSO and diluted to the final concentration with MBS. In control experiments, DMSO at concentrations of 1–3% did not significantly affect GABA-evoked Cl^- currents. Oocytes were exposed to drugs for 20 s (7–10 s were required to achieve equilibrium in the recording chamber). Intervals of 5–10 min were allowed between drug applications. GABA at the EC_{20} (concentration that induces a Cl^- current with an amplitude of 20±5% of the maximal response evoked by 1 mM GABA and which was determined for each oocyte at the beginning of the experiment) was used to produce the control response.

2.3.6. Pharmacology

Compounds **1** and **4** were suspended in distilled water with a drop of Tween 80 per 5 ml and injected in a volume of 3 ml, per kilogram of body weight. Control rats received an equivalent volume of vehicle. Rats were injected with pentylenetetrazole (55 mg/kg) or strychnine (5 mg/kg) at the indicated times after injection of vehicle, compounds **1** and **4**, respectively. The animals were observed for 15 or 30 min after injection of pentylentetrazole or strychnine, respectively, during which time of the latency of seizures and death were recorded. Data were analyzed by Fisher's exact probability test or Student's *t* test.

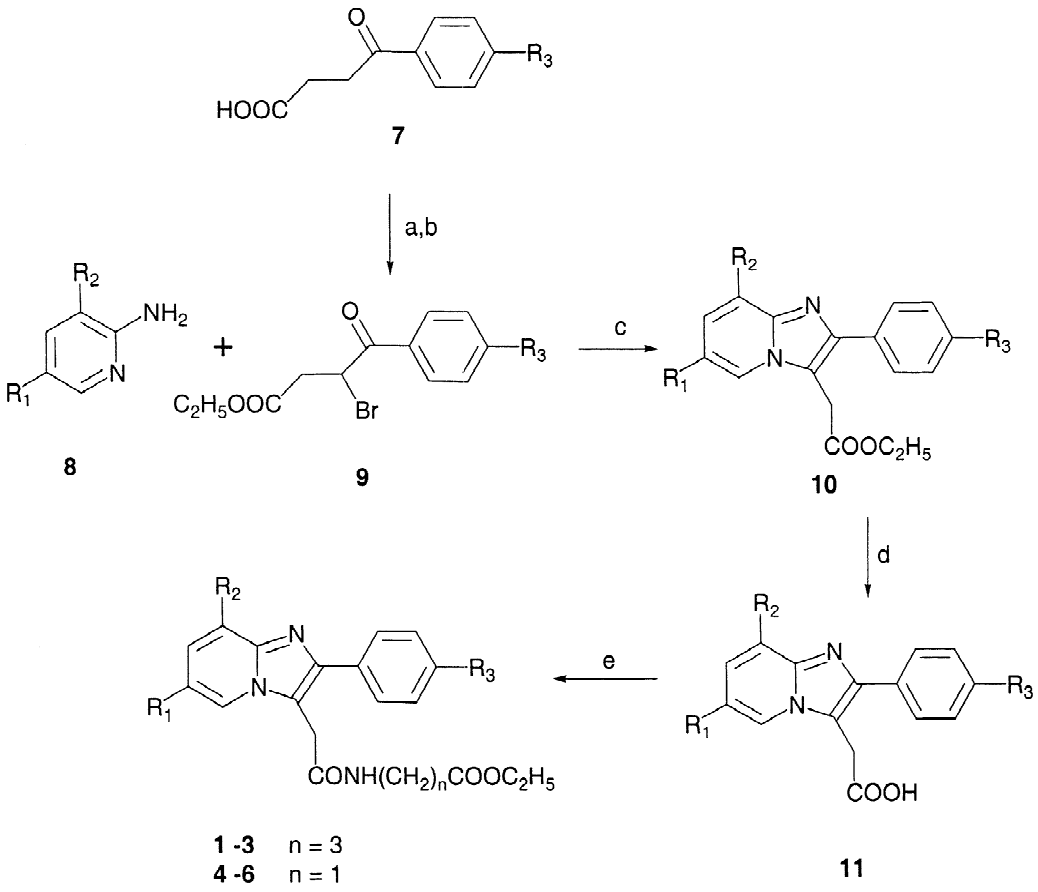
3. Results and discussion

3.1. Chemistry

According to Scheme 1, compounds **1**–**6** were prepared by condensing the appropriate carboxylic acid **11** with GABA- or Gly-ethyl esters·HCl in the presence of carbonyldiimidazole (CDI) as dehydrating agent. Compound **11**, in turn, was obtained by alkaline hydrolysis of esters **10** synthesized by condensation of suitably substituted 2-aminopyridines **8** with the appropriate bromoketoesters **9**. By these synthetic procedures, compounds **1**–**6** were prepared in moderate to good yield and were characterized by IR, ^{1}H-NMR and mass spectroscopy. Table 1 reports the structures and some physicochemical characteristics of compounds **1**–**6**.

*3.2. Lipophilicity and computational approach to estimate BBB penetration by compounds **1**–**6***

To be useful, compounds acting at the CNS level should possess good BBB penetration properties. Such a characteristic is roughly related to the compound's overall lipophilicity, which is reflected by its partition coefficient (log *P*) (Bodor and Buchwald, 1999). Therefore, the log *P* values between *n*-octanol and water of compounds **1**–**6** were calculated by using the CLOGP computer program and measuring retention in RP-HPLC (Table 1). A satisfactory correlation was found between the calculated log *P* values and the capacity factors log *k′*, and this analysis indicated a linear relationship with acceptable statistics ($n=6$, $r^2=0.89$, $s=0.18$). Log *P* values of 2.1 are generally considered optimal for penetration of the BBB (Norinder and Haeberlein, 2002). As can be seen from Table 1, compounds **1**–**6** possess log *P* values higher than 2.1 (i.e. in the range 3.73 –5.03) and appeared to be lipophilic enough



a) EtOH, H^+ b) Br_2 c) DMF reflux d) NaOH e) $^+NH_3CH_2CH_2CH_2COOC_2H_5$ Cl^- or $^+NH_3CH_2COOC_2H_5$ Cl^- /carbonyl diimidazole (CDI).

Scheme 1.

to cross the BBB. To gain further information on the BBB penetration properties of compounds **1**–**6** by passive transport, additional computational studies were carried out. Recently, several attempts to correlate BBB penetration with physicochemical parameters have been reviewed (Norinder and Haeberlein, 2002). It has been made clear that the octanol–water partition coefficient is an important factor, although in itself it correlates poorly with the log C_{brain}/C_{blood} (log BB). This ratio is a measure of the degree of BBB penetration. Experimental values of log BB published to date cover a range of about -2.00 to $+1.00$. Compounds with log BB >0.3 cross the BBB readily, while compounds with log BB <-1.0 are poorly distributed to the brain. In addition to log *P*, the importance of a molecular size descriptor has been shown, as well as the need to include a descriptor relating to hydrogen bond formation (Van de Waterbeemd et al., 1998). To readily estimate BBB penetration by compounds **1**–**6**, we used the model of Clark (1999) to predict the log BB of compounds **1**–**6**. This model relates log BB to polar surface area (PSA, defined as the surface area in $Å^2$ occupied by nitrogen and oxygen atoms and polar hydrogens connected to these heteroatoms) and calculated log *P* (Clog *P*) according to the equation: log BB $=-0.0148$ (± 0.001)PSA $+0.152$ (± 0.036)Clog $P+0.139$ (± 0.073). Calculation of the polar surface area of compounds **1**–**6** was performed using the MAREA software (Stenberg et al., 1999). As can be seen from the data reported in Table 1, on the basis of this computational approach, significant brain penetration can be predicted for compounds **1**–**6**.

3.3. In vitro studies

3.3.1. Radioligand binding assays

$GABA_A$ receptors are allosterically modulated by several types of compounds, including ethanol, benzodiazepines, barbiturates and neuroactive steroids (Majewska et al., 1986). Moreover, there is overwhelming evidence that activation of peripheral benzodiazepine receptors (PBR) stimulates the synthesis of neuroactive steroids (Giesen-Crouse, 1993). Apart from their pharmacological and biological functions, PBRs also differ from central benzodiazepine receptors (CBR) in their subcellular location in the brain, being present in glial cells (Giesen-Crouse,

1993). Nevertheless, PBR and CBR receptors share a common role in the CNS, where both modulate GABA-ergic transmission, PBRs doing so indirectly, by influencing neurosteroidogenesis, while CBRs act directly. It should be noted that alpidem interacts with both CBRs and PBRs. Previous works from these laboratories (Trapani et al., 1997, 1999) pointed out that substitution of the alpidem at position 8 with lipophilic substituents and the presence of one chlorine atom at the *para* position of the phenyl ring at C(2) are key structural features for high binding affinity and selectivity toward PBRs.

Evaluations to assess the interaction of compounds **1**–**6** with the GABA/benzodiazepine receptor complex at central (CBR) and peripheral (PBR) level were performed by measuring their ability to inhibit [^{3}H]-flunitrazepam and [^{3}H]-PK 11195 (Le Fur et al., 1983) binding to membrane preparations arising from the cerebral cortex. The reference compound in these binding assays was zolpidem and the measured binding affinities for CBR and PBR are shown in Table 2. As can be seen, compounds **1**–**6** displayed low affinity and selectivity for PBR. Only compounds **1** and **4** (i.e. the 8-unsubstituted imidazopyridine conjugates of GABA and Gly, respectively) possess a significant binding affinity towards CBR. It should also be noted that the acid **11** ($R_1 = R_3 = Cl$, $R_2 = H$) displayed very low ability to displace [^{3}H]-flunitrazepam from the cerebral cortex (IC_{50} 7 μM). These binding results suggest that the most active compounds (i.e. **1** and **4**) interact at the central (neuronal) but not at the peripheral (glial) receptors.

As shown in Table 2, compounds **1**–**6** were also evaluated for their ability to modulate the specific binding of [^{3}H]-GABA to washed membrane preparations from the rat cerebral cortex. The reference compound in this binding assay was abecarnil and the measured binding affinities for [^{3}H]-GABA are shown also in Table 2. Among the GABA-analogues **1**–**3**, only compound **1** enhanced the specific binding of [^{3}H]-GABA (about 30% above the control value), whereas the reference compound showed no effect (Table 2). Compounds **2** and **3** displayed a decrease in [^{3}H]-GABA binding modulation. Among the Gly-analogues, compound **4** showed a significant increase in [^{3}H]-GABA binding, even though in a concentration independent manner. Compounds **5** and **6** displayed little significant increase in [^{3}H]-GABA binding. Compounds **1** and **4**, however, did not show any direct action (GABA-mimetic) on chloride currents even at high concentrations (100 mM), thus confirming that their effects are modulatory in nature. On the whole, results from this in vitro test are consistent with those observed in benzodiazepine receptor binding assays, pointing to analogues **1** and **4** as the most active compounds.

Among the Gly-analogues, compound **4** was chosen to evaluate its ability to interact with the strychnine-sensitive Gly-binding sites. It was found that this compound failed to inhibit [^{3}H]-strychnine binding at the two concentrations tested (i.e. 1 μM and 10 μM).

3.3.2. Electrophysiology in Xenopus oocytes

Compounds **1**–**6** were evaluated both for their effects at human recombinant $GABA_A$ receptors, in order to determine their capacity to interact at CBR. As summarized in Table 3, Cl^- currents elicited by GABA at $GABA_A$ receptors were enhanced by compounds **1** and **4**, with maximal effects observed at 10 μM (111.8 and 80, respectively). Compounds **2, 3, 5** and **6** failed to produce statistically significant changes in the amplitude of GABA-elicited currents. It was also proved that acid **11** ($R_1 = R_3 = Cl$, $R_2 = H$) displayed a much lower ability to enhance GABA-evoked currents compared to compound **1** (data not shown). The ability of compounds **1** and **4** to inhibit GABA-induced Cl^- currents was flumazenil-sensitive (Table 3), indicating that the effect is mediated by the benzodiazepine recognition sites.

3.4. In vivo studies

The next aspect of these studies was to examine some in vivo properties of compounds **1** and **4**. Compound **1**

Table 2
Affinities of compounds **1**–**6** for CBR and PBR and modulatory action at [^{3}H]-GABA binding

Compound	IC_{50} (nM)[a]		[^{3}H]-GABA binding[a]			
	CBR	PBR	1 μM	10 μM	30 μM	100 μM
1	60	2600	+17	+30±3[b]	+27	+30
2	$>10^4$	57 000	−70	−51	−15	
3	$>10^4$	5500	−25	−18	−16	
4	150	1080	+26±8.8[c]	+34±4.5[c]		+46±0[b]
5	$>10^4$	6700	+16	+12		+12
6	$>10^4$	1600	+14	+5		+18
11 ($R_1 = R_3 = Cl$, $R_2 = H$)	7000	–				
Zolpidem	26	–				
Abecarnil			−5	−2	−13	

[a] Mean of two determinations. Relative standard deviation (R.S.D.) values were less than 8%.
[b] Mean of two experiments±standard deviation.
[c] Mean of three experiments±standard deviation.

Table 3
Modulatory action of compounds **1**–**6** at human α1β2γ2L $GABA_A$ receptors expressed in *Xenopus Laevis* oocytes[a]

Compound	0.1 μM	0.3 μM	1 μM	3 μM	10 μM
1	26.9±9.5	41.2±18.4	59.2±10.5	91.2±10.9 (13.6±3)[b]	111.8±17.7
2	15.0±6.1	10±4.0	6.1±6.0	12.0±5.0	3.2±3.2
3	−0.14±8.6	4.84±5.7	3.5±4.7	0.48±1.2	9.9±4.0
4	−4.95±18.9	15.8±12.7	44.9±11.5	70.9±8.7 (5±30)[b]	81.8±6.49
5	−3.3±13.8	3.9±8	2.5±2.3	11.4±9	6.3±12.3
6	−13.7±18.6	−0.7±3.1	9.3±2.4	21.2±4.1	15.8±7.8

[a] Values represent the potentiation of Cl^- currents induced by GABA and are means (n=5–8 different oocytes)±standard deviation.
[b] Values observed in the presence of flumazenil at 1 μM.

significantly antagonized pentylenetetrazde-induced seizures in rats. The time course of its anticonvulsant activity is shown in Table 4. Compound **1** administered 30 min before pentylenetetrazole decreased the number of animals showing convulsions, and completely protected animals from death. These protective effects were still evident, although not significant, 90 min after administration of compound **1**, while no differences were found between animals that had received the drug 120 min before pentylenetetrazole and controls. As for the times of the latency of seizures they were highest at 90 min after administration of compound **1**, and it is indicative of a prolonged pharmacological action. As expected, administration of GABA at a dose of 150 mg/kg, 30 min before pentylenetetrazole, failed to antagonize convulsions.

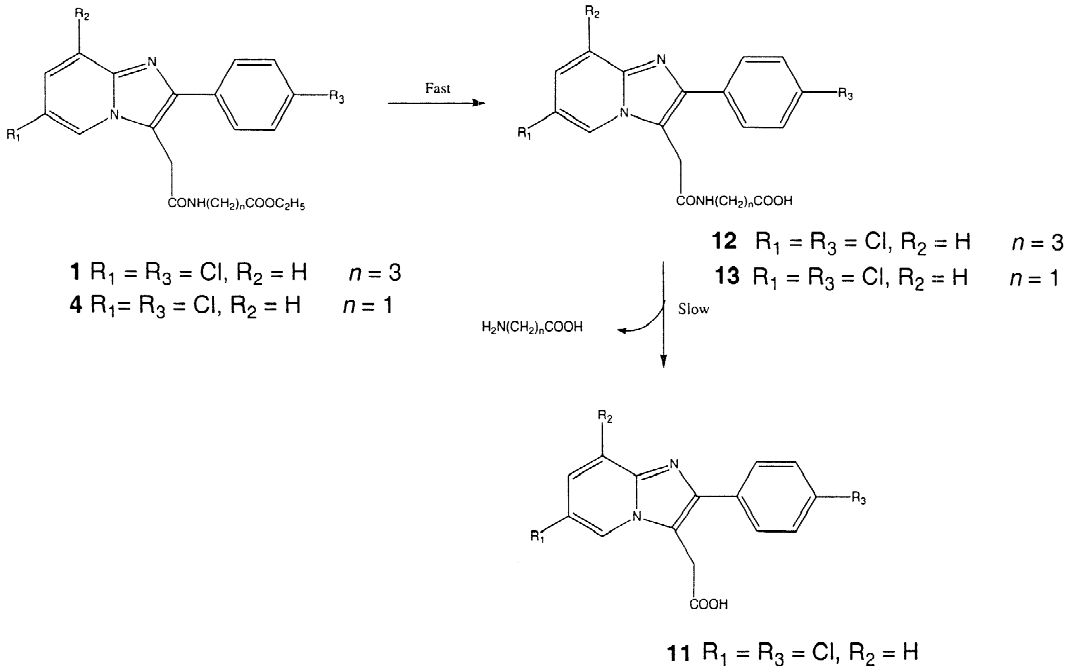
Administration of compound **4** at the dose of 150 mg/kg failed to antagonize convulsions induced by administration of strychnine (Table 4), and this is consistent with the corresponding binding assay. The times of the latency of convulsions were highest at 120 min after administration of compound **4** suggesting again a prolonged action in this pharmacological test.

To explain the effects of the most active compounds **1** and **4**, two hypotheses may be put forward: (i) a mechanism whereby these alpidem analogues remain intact and act as true benzodiazepine receptor ligands regulating GABA interaction by allosteric modulation; (ii) a prodrug mechanism whereby, once at the brain, **1** and **4** undergo chemical or enzymatic hydrolysis at the ester group to give compound **12** and **13**, respectively, or both at the ester and amide group to give the corresponding neurotransmitters and **11** (Scheme 2). Due to the fact that the amide bond is known to be quite stable in vivo, formation of **11** might be slow (Scheme 2). Both the proposed mechanisms also may work in combination. The prodrug mechanism may be proposed taking into account that **1** and **4,** although stable enough in solution buffered at pH 7.4, were found to undergo a rapid cleavage in dilute rat serum at 37 °C (data not shown). The delayed pharmacological effects observed for compounds **1** and **4** may be explained by the prodrug hypothesis. This last approach, indeed, is a well-established strategy for obtaining sustained release (Wermuth et al., 1996). On the other hand, *N*-benzoyl or *N*-pivaloyl, or *N*,*N*-phthaloyl derivatives of GABA as well as conjugation products of valproic acid and GABA or Gly are examples of compounds able to penetrate the BBB, most of them

Table 4
Anticonvulsant activity of **1** and **4** in the pentylenetetrazole and strychnine tests over time after i.p. administration of 150 mg/kg of compound to rats

Test	Compound	Test time (min)	Convulsions latency[a] (s)	Convulsions no. of animals	Death latency (s)[a]	Death no. of animals
Pentylenetetrazole	Vehicle		93±6	15/15	351±71	8/15
(55 mg/kg)	**1**	30	107±12	2/6[b]		0/6[c]
	1	90	139±26	5/8	240	1/8
	1	120	115±13	8/8	239±1	2/8
	GABA	30	95±8	8/8	331±55	6/8
Strychnine	Vehicle		166±34	12/12	558±108	10/12
(5 mg/kg)	**4**	30	185±46	6/6	672±66	6/6
	4	90	166±37	5/6	420±114	5/6
	4	120	343±77	6/6	450±96	6/6
	Glycine	120	197±26	6/6	486±102	6/6

Animals were treated with 150 mg/kg (i.p.) of **1** or **4** at the indicated times before pentylenetetrazole (55 mg/kg, i.p.) or strychnine (5 mg/Kg) and observed for at least 15 min (for compound **1**) and 30 min (for compound **4**).
[a] Data are means of two determinations±S.D.
[b] $P<0.01$.
[c] $P<0.025$ (Fisher's exact probability test).



Scheme 2.

exhibiting anticonvulsant effects (Wermuth et al., 1996; Bialer, 1999; Usifoh et al., 2001).

4. Conclusion

The main goal of the present paper was to show that alpidem analogues containing a GABA or Gly moiety, prepared by covalently coupling these neurotransmitters to an appropriate transport moiety (carrier group) represented by a lipophilic phenylimidazopyridine moiety, may gain access to the CNS by passive transport through the BBB and interact with their target site. Taken together, the in vitro results suggest that compounds **1** and **4** increases the function of the GABA–ionophore complex by interaction with the receptor. The observed in vivo anticonvulsant activity of compound **1** led us to conclude that it may be considered as a novel lipophilic GABA derivative, which deserves further consideration. This study demonstrated the feasibility of synthesizing useful anticonvulsants by coupling the amine function of the GABA with a phenylimidazopyridine portion. Moreover, the results presented in this article provide information regarding the subsequent design of phenylimidazopyridine derivatives of poorly BBB permeable drugs. In a companion paper (manuscript in preparation) we show the results of further studies aimed at elucidating both the chemical and enzymatic stability of compounds **1** and **4** and their brain distribution by microdialysis, an in vivo model for studying the penetration of BBB. These data provide insight into the mechanism behind the effects shown by **1** and **4**.

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