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European Journal of Pharmacology 516 (2005) 204 – 211

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Ethyl 2-(4-bromophenyl)-1-(2,4-dichlorophenyl)-1*H*-4-imidazolecarboxylate is a novel positive modulator of $GABA_A$ receptors

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Received 6 January 2005; received in revised form 28 April 2005; accepted 5 May 2005

Abstract

Ethyl 2-(4-bromophenyl)-1-(2,4-dichlorophenyl)-1*H*-4-imidazolecarboxylate (TG41) enhanced the binding both of γ-aminobutyric acid (GABA) and of flunitrazepam to rat cerebral cortical membranes. Electrophysiological recordings from *Xenopus* oocytes expressing various recombinant $GABA_A$ receptor subtypes revealed that TG41 enhanced the function of all receptor subunit combinations tested. The potency of TG41 at receptors containing α1, β2, and γ2L subunits was greater than that of alphaxalone, etomidate, propofol, or pentobarbital. The potency of TG41 was also greater at receptors containing α1 or α2 subunits than at those containing α4 and it was markedly higher at receptors containing β2 or β3 subunits than at those containing β1. This drug induced a reversible loss of the righting reflex in *Xenopus* tadpoles and it elicited hypnosis (5 mg/kg) after intravenous administration in rats. These results indicate that the pharmacological profile of TG41 is similar to that of general anesthetics which potentiate the activity of $GABA_A$ receptors containing the β2 or β3 subunit.

Keywords: γ-Aminobutyric acid; Transmembrane domain; Ethyl 2-(4-bromophenyl)-1-(2,4-dichlorophenyl)-1*H*-4-imidazolecarboxylate

1. Introduction

Type A receptors for the neurotransmitter γ-aminobutyric acid (GABA) are the most widely distributed inhibitory receptors in the higher regions of the vertebrate central nervous system (Bormann, 2000). These $GABA_A$ receptors are ligand-gated Cl^- channels and are composed of five transmembrane subunits. The binding of GABA to these receptors on neurons mediates fast inhibitory neurotransmission by inducing the opening of an intrinsic channel permeable to chloride and bicarbonate ions (Farrant and Nusser, 2005). This leads to an influx of anions and consequent cell hyperpolarization (Moss and Smart, 2001). Each $GABA_A$ receptor subunit possesses four putative transmembrane domains, a large intracellular loop between transmembrane domain 3 and transmembrane domain 4 that contains consensus sequences for phosphorylation by protein kinases, and a loop formed by two cysteine residues located in the amino-terminal extracellular domain (Bormann, 2000). $GABA_A$ receptor subunits identified to date include nineteen subunits: α1 –α6, β1–β3, γ1–γ3, ε, π, δ, θ, and ρ1–ρ3 (Farrant and Nusser, 2005). The most common $GABA_A$ receptors comprise two α subunits, two β subunits, and one γ subunit (Barnard et al., 1998). However, the large variety of subunits provides a basis for the possibility of substantial variability in the

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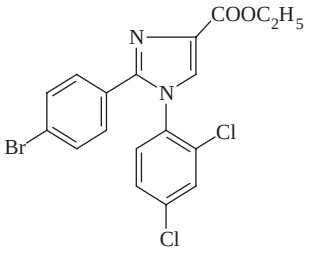
doi:10.1016/j.ejphar.2005.05.006



TG41

Fig. 1. Structure of TG41.

composition of $GABA_A$ receptors and such variability gives rise to receptor subtypes with different functional and pharmacological profiles (Rudolph et al., 2001).

$GABA_A$ receptors are modulated allosterically by various pharmacologically and chemically unrelated drugs, being important targets for benzodiazepines and imidazopyridines (Mohler et al., 2002), intravenous and volatile anesthetics (Mascia et al., 2000; Bali and Akabas, 2004), alcohols (Mihic et al., 1997), and neuroactive steroids (Lambert et al., 1995). In addition, GABA-mediated inhibitory neurotransmission is antagonized by picrotoxin and picrotoxinin as well as by several insecticides in current use such as lindane and endosulfan, all of which act as Cl^- channel blockers (Bloomquist, 2003). For a number of these compounds, including benzodiazepines, the intravenous anesthetic etomidate and the anticonvulsant loreclezole studies performed with recombinant $GABA_A$ receptors have revealed that their modulatory actions are subunit-specific (Wingrove et al., 1994; Sanna et al., 1997; Mohler et al., 2002). In addition, molecular biological studies have identified key amino acid residues that are either located in the binding sites or contribute to the modulatory actions of many of these drugs (Belelli et al., 1997; Mihic et al., 1997; Boileau and Czajkowski, 1999; Kucken et al., 2000).

Our biochemical and electrophysiological analyses have revealed that 1,2-diphenyl-imidazole derivatives are potent positive modulators of $GABA_A$ receptor function and have a potential general anesthetic action (Asproni et al., 2005). In the present study, we have investigated the action of ethyl 2-(4-bromophenyl)-1-(2,4-dichlorophenyl)-1*H*-4-imidazole-carboxylate (TG41) (Fig. 1) at recombinant $GABA_A$ receptors of various subunit compositions expressed in *Xenopus laevis* oocytes. Moreover, we have assessed the efficacy of this compound to induce loss of the righting reflex in both *Xenopus* tadpoles and rats.

2. Materials and methods

2.1. Materials

TG41 was donated by B. Asproni, University of Sassari, Italy, etomidate was a gift of Janssen, propofol was purchased from Aldrich, alphaxalone was obtained from Research Biochemical International, and pentobarbital was purchased from Sigma. [^{3}H]GABA and [^{3}H]flunitrazepam were purchased from New England Nuclear. All other drugs and materials were obtained from Sigma.

2.2. Assay of [^{3}H]GABA binding

Male Sprague–Dawley rats with body masses of 150–200 g were killed by decapitation and their brains were rapidly removed. The fresh cortical tissue was homogenized with a Polytron PT 10 disrupter for 30 s in 10 volumes of ice-cold water and the homogenate was centrifuged at 48,000 ×*g* for 10 min at 0 °C. The resulting pellet was washed once by resuspension and centrifugation in the original volume of a solution containing 20 mM potassium phosphate buffer (pH 7.4) and 50 mM KCl. The membrane pellet was then stored at −20 °C until binding analysis (1–5 days later). On the day of the assay, the membranes were thawed and washed a total of four times by resuspension and centrifugation in the same ice-cold solution. This extensive wash was performed with the intent of removing the endogenous GABA.

For the [^{3}H]GABA binding assay, the membranes were resuspended in 50 volumes of the same solution and 300 μl of membrane suspension (~300 μg of protein) were added to plastic minivials containing the drugs to be tested. The assay was performed in a final volume of 500 μl, started by the addition of [^{3}H]GABA (specific activity, 80–100 Ci/mmol) to a final concentration of 10 nM and stopped after incubation for 10 min at 0 °C by centrifugation at 48,000 ×*g* for 10 min at 0 °C. The pellet was gently washed with 4 ml of ice-cold water and then dissolved in 3 ml of scintillation fluid. The membrane-associated radioactivity was determined with a scintillation counter (TRI-CARB 2100TR, Packard). Drug stock solutions (20 mM) were prepared in dimethyl sulfoxide (DMSO). Binding data were corrected for nonspecific binding, which was determined in the presence of 1 mM GABA.

2.3. Assay of [^{3}H]flunitrazepam binding

Fresh cortical tissue isolated as above was suspended in 50 volumes of 50 mM Tris–HCl (pH 7.4) and homogenized with a Polytron PT 10 for 30 s and the homogenate was then centrifuged twice at 48,000 ×*g* for 10 min at 0 °C. The resulting pellet was resuspended in 50 volumes of the same buffer and used for the assay. [^{3}H]Flunitrazepam binding was determined in a final volume of 1 ml consisting of 200 μl of membranes (~200–400 μg of protein), 100 μl of 5 nM (final concentration 0.5 nM) [^{3}H]flunitrazepam (specific activity, 70–100 Ci/mmol), 5 μl of drug or solvent, and 695 μl of 50 mM Tris–HCl (pH 7.4). Incubations were performed for 60 min at 0 °C and the extent of [^{3}H]flunitrazepam binding was determined as described for [^{3}H]GABA binding. Drug stock solutions (20 mM) were prepared in DMSO. Binding data were corrected for nonspecific binding, which was determined in the presence of 5 μM diazepam.

2.4. Expression of human $GABA_A$ receptor subunits

A mixture of pCDM8-based vectors for the α1, α2, α4, β1, β2, β3, γ2L, or γ3 subunits of human $GABA_A$ receptors (total of 1.5 ng of DNA, comprising equal amounts of α, β, and γ subunit vectors) was injected into the animal pole of *X. laevis* oocytes as described (Colman, 1984) with the use of a microdispenser

(Drummond Scientific, Broomwall, PA). The injected oocytes were maintained at 15 °C in sterile modified Barth's solution [MBS: 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 $Ca(NO_3)_2$] supplemented with streptomycin (10 mg/l), penicillin (10,000 U/l), gentamicin (50 mg/l), theophylline (90 mg/l), and pyruvate (220 mg/l). Correct expression of an equal ratio of α, β and γ $GABA_A$ receptor subunits was confirmed using 1 μM diazepam as control. This concentration of diazepam potentiated the $GABA_A$ receptor function 96.2±11.2%.

2.5. Electrophysiology

Electrophysiological measurements were performed in oocytes 1–4 days after DNA injection. Oocytes were placed in a rectangular chamber (volume ~100 μl) and perfused at a rate of 1.7 ml/min with MBS at room temperature with the use of a roller pump (Cole-Parmer, Chicago, IL) and 18-gauge polyethylene tubing (Clay Adams, Parsippany, NJ). Oocytes were impaled at the animal pole with two glass electrodes (0.5–10 MΩ) filled with 3 M KCl and were clamped at −70 mV with the use an oocyte clamp (model OC725C; Warner Instruments, Hamden, CT). Currents were continuously plotted with a chart recorder (Cole-Parmer). GABA (Sigma, St. Louis, MO) was dissolved in MBS and applied to the oocytes for 30 s.

Oocytes were perfused with test drugs for 30 s either in the absence of GABA or in its presence at the EC_{10} (the concentration of GABA that induces a peak current equal to 10% of the maximal current elicited by 1 mM GABA). TG41, propofol and alphaxalone were first dissolved in DMSO at a concentration of 100 mM and then diluted in MBS to a final DMSO concentration not exceeding 0.3%, a concentration that did not affect the GABA receptor function. In each experiment, control responses were determined before and 10 min after application of drug.

2.6. Behavioral effects in tadpoles and rats

X. laevis tadpoles (43–50 days old) were maintained in an aquarium at 20–22 °C. For determination of loss of the righting reflex (LORR), 10 tadpoles were placed in separate beakers containing 300 ml of tap water with or without TG41 (0.01–30 μM). With the exception of the tap water control, all beakers contained DMSO at 0.1%, a concentration that did not affect animal behavior. Anesthesia was defined as the absence of a purposeful and sustained swimming response after inversion of the tadpole with a smooth glass rod. The number of anesthetized tadpoles was recorded every 10 min for up to 120 min, after which the tadpoles were returned to fresh tap water. Normal swimming activity was restored within 30 min.

The behavioral effects of TG41 were also evaluated in adult male Sprague–Dawley rats (body mass, 150–200 g) after intraperitoneal or intravenous injection. TG41 was suspended in physiological saline containing five drops of Tween 20 per milliliter and was then subjected to ultrasonic treatment for at least 1 h. Saline was used as vehicle. For intraperitoneal administration, TG41 was suspended in a volume of 3 ml per kilogram of body mass. Intravenous injection (2.5 ml/kg) was performed through a catheter that had been inserted in the jugular vein under anesthesia with equithesin 3 days before the experiment. Animals were observed 1 h after injection and anesthesia was defined as loss of the righting reflex.

Animal care and handling throughout the experimental procedures were in accordance with the European Communities Council Directive of November, 24, 1986 (86/609/EEC) and were approved by the local ethics committee.

3. Results

The effect of TG41 on the binding of [^{3}H]GABA to rat cerebral cortical membranes was assessed and compared with other drugs known as efficacious positive modulators of the $GABA_A$ receptor function such as the intravenous anesthetics alphaxalone, propofol and pentobarbital, and the benzodiazepine diazepam. TG41 (0.001–10 μM) increased specific [^{3}H]GABA binding with a potency and efficacy greater than those of pentobarbital, alphaxalone, propofol, or diazepam (Fig. 2A). Moreover, TG41 (0.001–10 μM) enhanced specific [^{3}H]flunitrazepam binding to rat cerebral cortical membranes again with a potency higher than those of alphaxalone and etomidate; however, the efficacy was higher than that of alphaxalone but the same as etomidate (Fig. 2B).Concentrations of TG41 higher than 0.1 μM resulted, however, in a reduced extent of potentiation giving a bell-shaped concentration–response curve. As previously shown (Concas et al., 1990), the

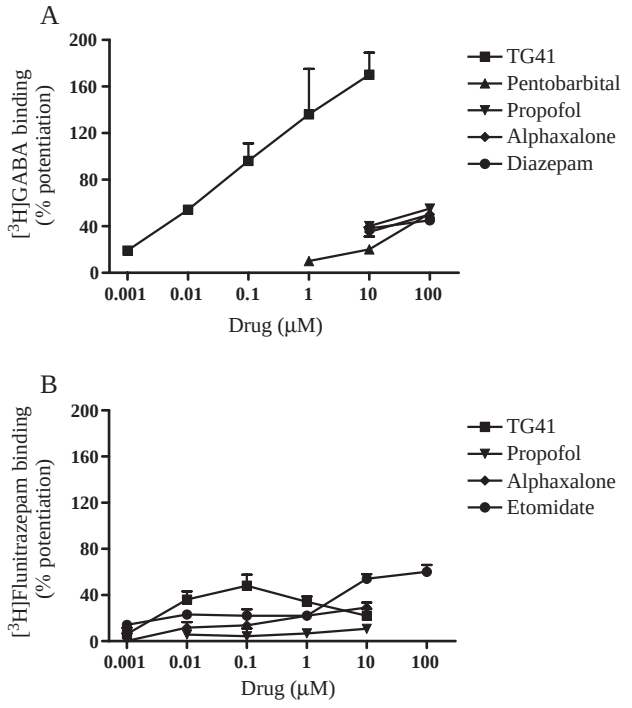


Fig. 2. Effects of TG41 and various other drugs on [^{3}H]GABA binding and [^{3}H]flunitrazepam binding to rat cerebrocortical membranes. (A) [^{3}H]GABA binding. The indicated concentrations of TG41, pentobarbital, propofol, alphaxalone, and diazepam were assessed for their effects on the specific binding of [^{3}H]GABA to frozen–thawed membrane preparations. Data are expressed as percentage potentiation of [^{3}H]GABA binding and are means±S.E.M of values from three independent experiments, each performed in triplicate. (B) [^{3}H]flunitrazepam binding. The indicated concentrations of TG41, propofol, alphaxalone, and etomidate were assessed for their effects on the specific binding of [^{3}H]flunitrazepam to freshly prepared membranes. Data are expressed as percentage potentiation of [^{3}H]flunitrazepam binding and are means±S.E.M of values from three independent experiments, each performed in triplicate.

general anesthetic propofol failed to modulate specific [^{3}H]flunitrazepam binding. Exposure of *X. laevis* oocytes expressing recombinant α1β2γ2L $GABA_A$ receptors to TG41 (0.01–10 μM) resulted in a marked concentration-dependent potentiation of inward Cl^- currents induced by GABA at the EC_{10} value (Fig. 3A). The potency of TG41 in this regard was again greater than those of other GABAergic modulators tested (Fig. 3B), with the EC_{50} values increasing according to the rank order of TG41 (0.13 μM)<alphaxalone (1.6 μM)<etomidate (3.17 μM)<propofol (11.2 μM)<pentobarbital (~50 μM).

We next assessed the selectivity of TG41 for different subtypes of $GABA_A$ receptors. Comparison of the modulatory effects of TG41 at α1β2γ2L, α2β2γ2L, and α4β2γ2L receptors revealed that the maximal potentiation of GABA-induced Cl^- currents by TG41 was similar at these three receptor subtypes (533%, 595%, and 664%, respectively); however, TG41 exhibited a greater potency at receptors containing the α1 or α2 subunits (EC_{50} of 0.7 μM) than at those containing the α4 subunit (EC_{50} of 6.4 μM) ($p<0.01$ vs. α1 or α2 subunits) (Fig. 4A). Comparison of the modulatory effects of TG41 at α1β1γ2L, α1β2γ2L, and α1β3γ2L receptors again revealed similar efficacies at these three receptor subtypes; the potency of TG41, however, was greater at receptors containing the β2 (EC_{50} of 0.19 μM) or β3 (EC_{50} of 0.24 μM) subunits than at those containing the β1 subunit (EC_{50} of 1.7 μM) ($p<0.01$ vs. β1 subunit) (Fig. 4B). However, in $GABA_A$ receptors containing β2 subunits we sometimes experienced a decrease in the enhancement of the $GABA_A$ receptor function induced by high (30 μM) concentrations of TG41. This decrease may be due to receptor desensitisation and has been noticed before with

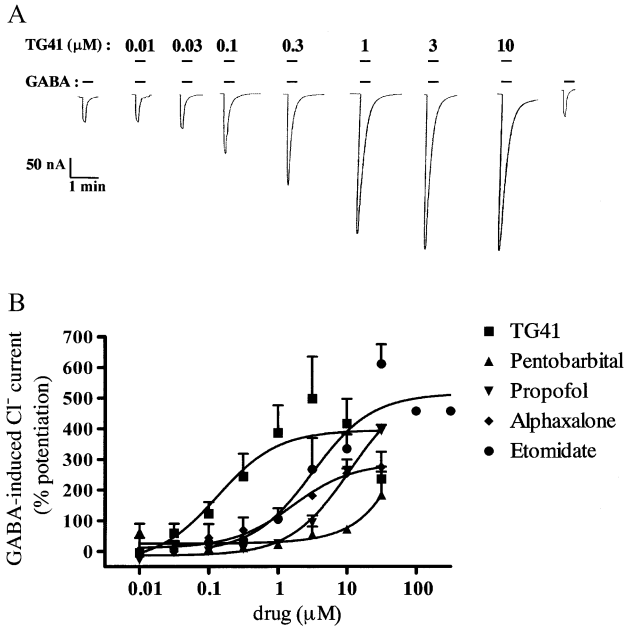


Fig. 3. Effects of TG41 and other drugs on GABA-induced Cl^- currents in *Xenopus* oocytes expressing recombinant α1β2γ2L $GABA_A$ receptors. (A) Representative tracings of Cl^- currents in an individual voltage-clamped oocyte induced by the application of GABA at the EC_{10} value in the absence or presence of the indicated concentrations of TG41. Horizontal bars indicate the duration of GABA or TG41 application. (B) Concentration–response curves for the effects of TG41, propofol, etomidate, pentobarbital, and alphaxalone on the Cl^- currents induced by GABA at the EC_{10} value. Data are expressed as percentage potentiation of the GABA response and are means±S.E.M of values obtained from 8 to 11 oocytes.

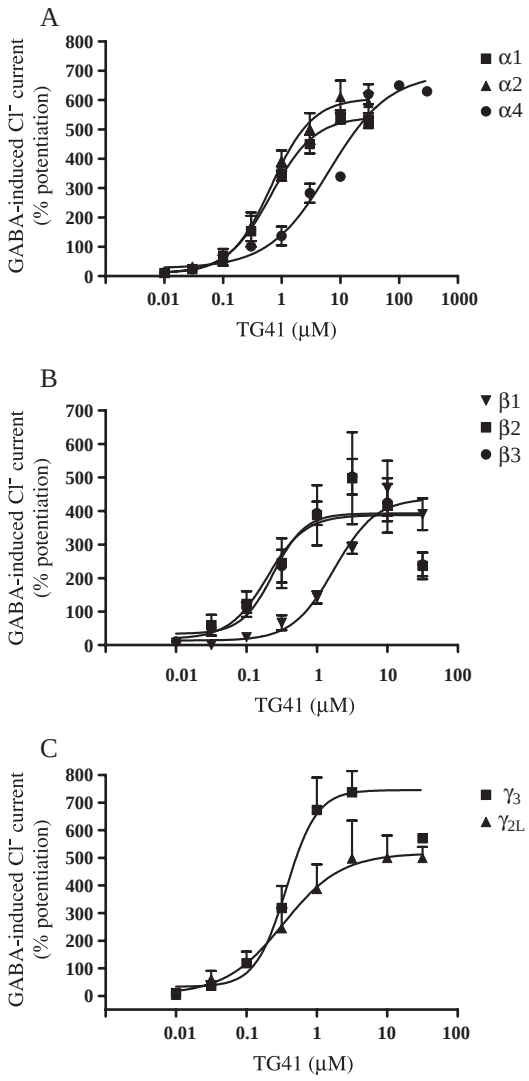


Fig. 4. (A) Concentration–response curves for TG41 modulation of GABA-induced Cl^- currents in *Xenopus* oocytes expressing α1β2γ2L, α2β2γ2L, or α4β2γ2L $GABA_A$ receptors. (B) Concentration–response curves for TG41 modulation of GABA-induced Cl^- currents in *Xenopus* oocytes expressing α1β1γ2L, α1β2γ2L, or α1β3γ2L $GABA_A$ receptors. (C) Concentration–response curves for TG41 modulation of GABA-induced Cl^- currents in *Xenopus* oocytes expressing α1β2γ2L or α1β2γ3 $GABA_A$ receptors. Data are expressed as percentage potentiation of the response induced by GABA at the EC_{10} value and are means±S.E.M of values from 8 to 14 oocytes.

compounds which are strong positive modulators of the GABA receptor function, such as steroids (Carter et al., 1997). This selectivity of TG41 for receptors containing β2 or β3 subunits is similar to that previously described for etomidate and loreclezole (Belelli et al., 1997; Wingrove et al., 1994). Finally, we compared the modulatory effects of TG41 at α1β2γ2L and α1β2γ3 receptors. Whereas TG41 enhanced GABA-induced Cl^- currents at these two receptor subtypes with a similar potency (EC_{50} of 0.3 μM), its maximal efficacy was greater at receptors containing γ3 (780%) than at those containing γ2L (500%) ($p<0.01$ vs. γ3 subunits) (Fig. 4C).

To determine if TG41 acts at the benzodiazepine binding site we next evaluate the ability of flumazenil to block the potentiation of the $GABA_A$ receptor function induced by TG41. In α1β2γ2L

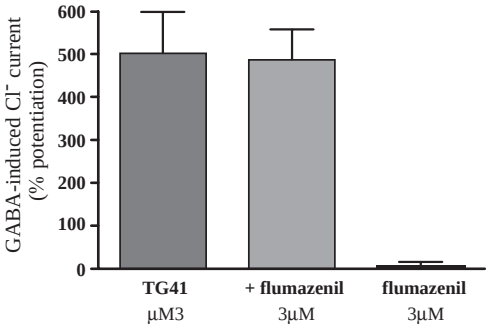


Fig. 5. Failure of flumazenil in modifying the modulation of GABA-induced Cl^- currents by TG41 in *Xenopus* oocytes expressing α1β2γ2L receptors. Data are expressed as percentage potentiation of the response induced by GABA at the EC_{10} value and are means ± S.E.M of values from 6 to 10 oocytes.

receptors 3 μM TG41 enhanced GABA-evoked Cl^- currents, however, this enhancement was not affected by flumazenil (3 μM) (Fig. 5).

At relatively high concentrations (3–10 μM), TG41 directly activated Cl^- flux in *Xenopus* oocytes expressing α1β2γ2L receptors (Fig. 6). Direct activation of Cl^- currents was also observed with related 1,2-diphenyl-imidazole derivatives (data not shown).

To evaluate the selectivity of TG41 for the $GABA_A$ receptor, we also examined whether this compound affected the function of two other ligand-gated ion channels: the strychnine-sensitive glycine receptor and the 5-HT_3 receptor. The function of both of these receptors is modulated by several general anesthetics and the inhibitory glycine receptor shares many molecular and functional features with the $GABA_A$ receptor. We expressed 5-HT_{3A} receptors or glycine (α1 homomeric) receptors in *X. laevis* oocytes and compared the effects of TG41 with those of etomidate. TG41 induced a concentration-dependent inhibition of serotonin-induced current in oocytes expressing human 5-HT_{3A} receptors whereas etomidate had no significant effect (Fig. 7A). Neither TG41 nor etomidate affected glycine-evoked Cl^- currents in oocytes expressing human glycine receptors (Fig. 7B).

The behavioral effects of TG41 were assessed in *X. laevis* tadpoles and rats. TG41 induced a reversible loss of the righting reflex in tadpoles with an EC_{50} of 0.28 μM (Fig. 8). In rats, intravenous administration of TG41 at a dose of 5 mg/kg induced loss of the righting reflex within 8 s and for a duration of 28 min

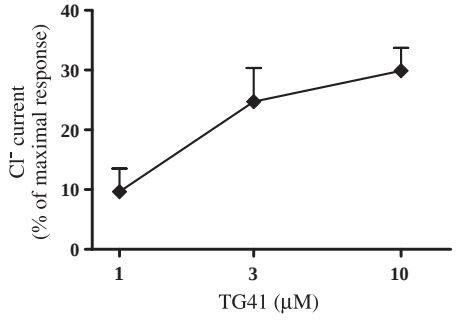


Fig. 6. Direct induction by TG41 of Cl^- currents in *Xenopus* oocytes expressing α1β2γ2L $GABA_A$ receptors. Data are expressed as a percentage of the current response elicited by 10 mM GABA and are means ± S.E.M of values from 10 to 16 oocytes.

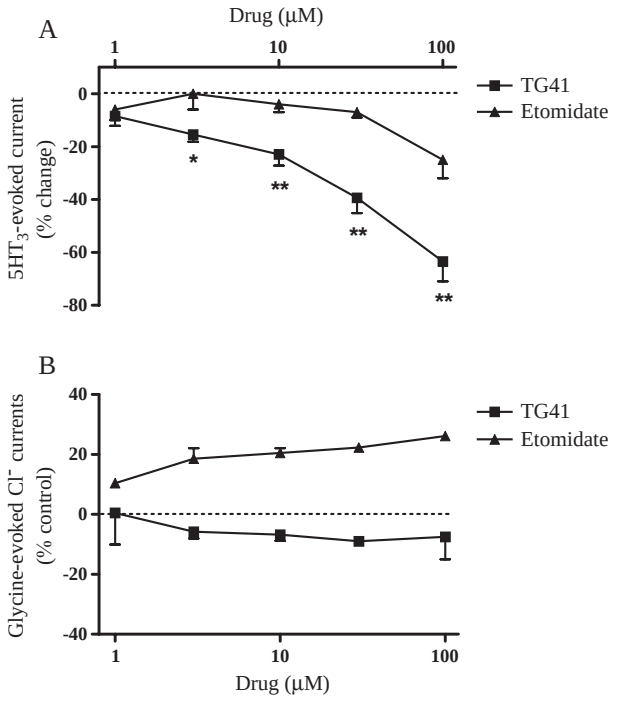


Fig. 7. Effects of TG41 and etomidate on ligand-induced current responses in *Xenopus* oocytes expressing 5-HT_{3A} receptors (A) or glycine (α1 homomeric) receptors (B). Data are expressed as percentage change relative to the control response obtained with serotonin (A) or glycine (B) at the EC_{10} value and are means ± S.E.M of values from five to eight oocytes from two or more batches. $^{**}P<0.001$ or $^{*}P<0.05$ vs. the control response in the absence of TG41 by analysis of variance followed by post hoc test.

(Table 1). However, intraperitoneal administration of TG41 (30 mg/kg) had no effect. In contrast, the anesthetic etomidate induced loss of the righting reflex by intraperitoneal injection at a dose of 30 mg/kg, as previously shown by Reynolds et al. (2003), and by intravenous injection at a dose of 3 mg/kg. Doses of etomidate and TG41 chosen for the intravenous administration were roughly equimolar.

4. Discussion

The aim of this study was to investigate the pharmacological, electrophysiological, and behavioral properties of

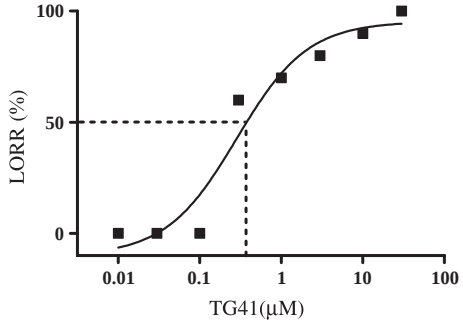


Fig. 8. Anesthetic action of TG41 in *X. laevis* tadpoles. The anesthetic effect of TG41 (0.01–30 μM) was assessed from loss of the righting reflex (LORR) among 10 tadpoles for each drug concentration.

Table 1
Behavioral effects of TG41 and etomidate in rats

Drug	Time to onset	Duration (min)	Effect
TG41 (30 mg/kg, i.p.)			No effect
TG41 (5 mg/kg, i.v.)	8 s	28	LORR
Etomidate (30 mg/kg, i.p.)	60 min	60	LORR
Etomidate (3 mg/kg, i.v.)	28 s	5	LORR

Data are means of values from 6 rats. LORR, loss of righting reflex.

TG41, a novel 1,2-diphenyl-imidazole derivative. Our results demonstrate that TG41 is a potent positive modulator of $GABA_A$ receptor function.

We found that TG41 enhanced the [^{3}H]flunitrazepam binding to rat cerebrocortical membranes with a potency higher than that of the intravenous anesthetic alphaxalone. However, this potentiation occurs in a bell-shaped fashion with concentrations of TG41 higher than 0.1 μM decreasing the enhancement of [^{3}H]flunitrazepam binding. It is possible that TG41 at high concentrations induces a change in the $GABA_A$ receptor conformation that in turn stabilizes the closed state of the receptor. In addition, the potency of TG41 was greater than that of propofol, pentobarbital, or alphaxalone with regard to the enhancement of [^{3}H]GABA binding to cerebral cortical membranes. These results suggest that TG41 may interact with a distinct binding site on the $GABA_A$ receptor complex. Consistent with our biochemical data, electrophysiological recordings demonstrated that TG41 potentiated GABA-evoked Cl^- currents in *Xenopus* oocytes expressing recombinant α1β2γ2L $GABA_A$ receptors. Again, the potency of TG41 was greater than that of propofol, alphaxalone, etomidate, or pentobarbital in this regard. TG41 did not exhibit a marked selectivity for $GABA_A$ receptors containing a specific α subunit, although the EC_{50} values for potentiation of receptor function were slightly smaller for receptors containing α1 or α2 than for those containing α4. Given that the α subunits of $GABA_A$ receptors mediate many pharmacological actions of benzodiazepines (anxiolysis, sedation, hypnosis, and amnesia) and that $GABA_A$ receptors that contain the α4 subunit are insensitive to benzodiazepines, our electrophysiological data provide further support for the notion that the pharmacological profile of TG41 differs from that of benzodiazepines.

TG41 showed a strong selectivity for $GABA_A$ receptors that contain the β2 or β3 subunit compared with those that contain the β1 subunit. Substitution of the β2 or β3 subunit with β1 thus resulted in a marked increase in the EC_{50} value for the enhancement of $GABA_A$ receptor function by TG41. A similar β subunit selectivity has been demonstrated for the intravenous anesthetic etomidate (Sanna et al., 1997). The difference in the etomidate sensitivity of $GABA_A$ receptors containing different β subunits has been attributed to a single amino acid located in transmembrane domain 2. This residue is asparagine-289 in the β2 or β3 subunit, whereas the corresponding amino acid in the β1 subunit is serine. Replacement of asparagine-289 in the β2 or β3 subunit with serine thus resulted in a marked reduction in both the GABA-modulatory and GABA-mimetic effects of etomidate (Belelli et al., 1997). Behavioral studies performed with genetically modified mice have confirmed the importance of this amino acid and therefore that of the β subunit, not only for the action of etomidate but also for that of the general anesthetic propofol (Jurd et al., 2003). In addition, methionine-286 in transmembrane domain 3 of β2 subunit appears to be important for propofol action at $GABA_A$ receptors (Krasowski et al., 2001; Bali and Akabas, 2004), possibly contributing to a binding site for this anesthetic (in these reports the amino acid sequence starts after the putative signal peptide of 24 residues). The importance of the β subunit of the $GABA_A$ receptor for the action of intravenous anesthetics was also indicated by a recent behavioral study performed with genetically modified mice showing that the β2 subunit mediates the sedative properties of these drugs whereas the β3 subunit mediates loss of consciousness (Reynolds et al., 2003). Together, these various observations suggested that TG41 might exert an anesthetic action.

TG41 also showed a selectivity for $GABA_A$ receptors containing the γ3 subunit compared with those that contain the γ2L subunit. The maximal efficacy of TG41 in potentiating GABA-evoked Cl^- currents was thus greater at α1β2γ3 receptors than at α1β2γ2L receptors. A similar γ subunit selectivity has been demonstrated for the direct effects of propofol and some propofol derivatives at $GABA_A$ receptors (Sanna et al., 1999).

At higher concentrations, these compounds were more effective in eliciting Cl^- currents in the absence of GABA at receptors containing the γ3 subunit than at those containing γ2L. Similar to many general anesthetics, TG41 also directly activated $GABA_A$ receptors in the absence of GABA. Direct activation of α1β2γ2L receptors by TG41 was apparent at concentrations as low as 3 μM; the effect of TG41 at this concentration was 24% of the maximal GABA response, which is similar to that of 100 μM propofol (≈20% of the maximal GABA response) previously determined in *Xenopus* oocytes expressing α1β1γ2L receptors (Wafford et al., 1996).

TG41 did not affect glycine-evoked Cl^- currents in *Xenopus* oocytes expressing glycine receptors consisting of the α1 subunit; etomidate also had no effect on glycine receptor function in this assay, consistent with previous observations (Mascia et al., 1996; Pistis et al., 1997). In contrast, TG41 inhibited the function of 5-HT_{3A} receptors. This inhibition occurred at concentrations (≥3 μM) higher than those found to enhance $GABA_A$ receptor function, however, indicating that TG41 is relatively selective for $GABA_A$ receptors. Etomidate did not affect 5-HT_{3A} receptor function in our assay, although it significantly reduced the cation permeability of 5-HT_3 receptors in NIE-115 cells (Parker et al., 1996).

Assessment of loss of the righting reflex in *X. laevis* tadpoles indicated that TG41 behaves as an anesthetic. The

EC_{50} for the induction of this behavioral effect was ~0.3 μM, a concentration lower than those found to be effective for other intravenous anesthetics (propofol, alphaxalone, etomidate, and pentobarbital) that act at $GABA_A$ receptors (Mascia et al., 1996). In addition, at this concentration, TG41 markedly enhanced $GABA_A$ receptor function but did not affect the activities of 5-HT_{3A} or glycine receptors. These observations thus suggest that the loss of the righting reflex induced by TG41 may be mediated solely by the action of this drug at $GABA_A$ receptors.

Finally, in contrast to etomidate, TG41 administered intraperitoneally to rats did not induce anesthesia. It did, however, elicit anesthesia after intravenous injection in rats with a potency similar to that of etomidate. The failure of TG41 to induce anesthesia after intraperitoneal administration might be attributable to hydrolysis of the drug by esterases in plasma. Indeed, the parent compound of TG41, which lacks an ester group, modulates $GABA_A$ receptor function with a low potency and efficacy (Asproni et al., submitted). In addition, further metabolism of TG41 by hepatic decarboxylases may render it completely inactive. It is unlikely that the inability of TG41 to induce anesthesia after intraperitoneal administration in rats is due to poor absorption because its lipophilicity is greater than that of etomidate.

In conclusion, our results demonstrate that TG41 is a potent and selective modulator of the $GABA_A$ receptor and may act on a site common to general anesthetics but different from benzodiazepine recognition site as the potentiation of the $GABA_A$ receptor function by TG41 was not affected by the benzodiazepine receptor antagonist flumazenil. Both biochemical and electrophysiological data indicate that TG41 potentiates $GABA_A$ receptor function with a higher potency and efficacy than do other intravenous anesthetics known to enhance GABAergic transmission. In addition, the characteristics of TG41 action at recombinant $GABA_A$ receptors are similar to those of the action of the intravenous anesthetic etomidate. Our behavioral experiments performed with tadpoles and rats further confirm that TG41 has the pharmacological profile of an injectable anesthetic.

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