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Anticonvulsant, anxiolytic, and non-sedating actions of imidazenil and other imidazo-benzodiazepine carboxamide derivatives

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ABSTRACT

Recent evidence suggests that α1-containing $GABA_A$ receptors mediate the sedative, amnestic, and to some extent the anticonvulsant actions of non-selective benzodiazepine (BZ) receptor ligands, such as diazepam (DZ). Anxiolytic and in part, anticonvulsant actions of BZ ligands are mediated by α2-, α3-, and α5-containing $GABA_A$ receptors. This has resulted in increasing interest in developing BZ ligands with selective actions at $GABA_A$ receptors, including α2-, α3-, and α5-subunits, but devoid of efficacy at α1-containing receptors. To refine their spectrum of pharmacological actions, efforts are being made to minimize unwanted effects such as sedation, amnesia, and tolerance liabilities. A prototype for such BZ ligands is imidazenil (IMD), an imidazo-benzodiazepine carboxylic acid derivative that elicits potent anticonvulsant and anxiolytic actions at doses virtually devoid of sedative, cardio-respiratory depressant and amnestic effects, and anticonvulsant tolerance liability. To define the pharmacological profile of IMD and its derivatives, we compared the anticonflict (anxiolytic), anti-proconflict (antipanic), anti-bicuculline (BIC), and maximal electroshock seizure (MES) effects, and the suppression of locomotor activity by imidazo-benzodiazepine carboxylic acid derivatives to those of DZ and bretazenil (BTZ). We report here that IMD and one of its derivatives (RO 25-2775) possess dose-dependent anticonflict, anti-proconflict, and anti-BIC actions but failed to suppress locomotor activity. Like DZ, the other IMD derivatives (enazenil, RO 25-2776, and RO 25-2847) not only elicit dose-dependent anticonflict, anti-proconflict, anti-BIC, anti-MES effects but also suppress locomotor activity. In contrast, none of the IMD derivatives studied shows any similarity to BTZ, which elicits anticonflict, anti-proconflict actions and suppresses locomotor activity but is virtually inactive against BIC-induced tonic–clonic convulsions.

1. Introduction

The facilitation of γ-aminobutyric acid (GABA)-mediated neurotransmission in the central nervous system (CNS) by benzodiazepines (BZs) and their congeners is a fundamental outcome of their pharmacotherapeutic action, which may be useful in the treatment of psychiatric and neurological disorders, such as generalized anxiety disorders, psychotic and cognitive disorders, sleep disturbances, muscle spasms, and various aspects of complex seizure disorders. The BZ recognition site through which BZs and their congeners exert their positive allosteric modulatory action on GABA-gated Cl^- currents is located on $GABA_A$ receptors. These receptors are also targets for other types of psychoactive drugs such as barbiturates, anesthetics, and neurosteroids.

$GABA_A$ receptors are heteroligomeric pentameric transmembrane neuronal proteins that comprise GABA-gated Cl^- channels (Wisden and Seeburg, 1992; Macdonald and Olsen, 1994; Barnard, 1995). The heterogeneity of these receptors results from an association of different multiple variant polypeptide subunits (α1-α6, β1–β3, γ1–γ3, ρ1–ρ3, δ, ε, θ), which may characterize the functional aspects of $GABA_A$ receptor subtypes. The majority of these $GABA_A$ receptors include various α-, β-, and γ-subunits in their assembly (Barnard et al., 1998). Using gene-targeting approaches (Rudolph et al., 1999; Mckernan et al., 2000) in mice, it has been demonstrated that $GABA_A$ receptors, including the α1 subunit, mediate the sedative, amnestic, and to some extent the anticonvulsant actions of non-selective BZ receptor ligands such as DZ. The anxiolytic and in part, the anticonvulsant actions of BZs are mediated by α2-, α3-, and α5-containing $GABA_A$ receptors (Low et al., 2000; Mckernan et al., 2000; Rudolph et al., 2001; Atack et al., 2006). The subunit-selective positive allosteric modulators of GABA action at $GABA_A$ receptors by various BZ ligands that act on α2, α3, and α5 subunit complexes (i.e., imidazenil, and the Merck compound MK-0777, for details see Lewis et al., 2008; Guidotti et al., 2005) but are devoid of action on α1-containing $GABA_A$ receptors have been considered good candidate drugs to alleviate the downregulation of GABAergic transmission underlying the behavioral

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and electrophysiological alterations in individuals with schizophrenia and epileptic disorders (Lewis et al., 2008; Guidotti et al., 2005).

The advances in the selectivity and/or specificity of BZ actions at different $GABA_A$ receptor subtypes have become useful in the design of a "second generation" of BZ ligands that have selective actions at defined $GABA_A$ receptor subtypes. It is hoped that these ligands will have a refined spectrum of pharmacological actions while minimizing unwanted effects such as sedation, amnesia, and tolerance liabilities.

In previous work, we described the pharmacological profile of imidazenil (IMD) (Giusti et al., 1993), a prototypic "second generation" imidazo-benzodiazepine carboxylic acid benzodiazepine. Subsequently, we demonstrated that this imidazo-benzodiazepine carboxamide elicits potent anticonvulsant and anxiolytic actions at doses that do not produce sedation, cardio-respiratory depression, anticonvulsant tolerance liability, or amnestic effects (Auta et al., 1994, 1995, 2000, 2005, 2008; Impagnatiello et al., 1996; Costa et al., 2001, 2002; Guidotti et al., 2005).

The present studies were designed to compare the pharmacological profile of imidazo-benzodiazepine carboxylic acid derivatives to that of: 1) DZ, a prototyped non-selective and full positive allosteric modulator (FPAM) of GABA action at α1, α2, α3, and α5-containing $GABA_A$ receptors (Mohler et al., 2001; Lagrange et al., 2007; Guidotti et al., 2005; Costa et al., 2002) with no intrinsic efficacy at α4 and α6-containing $GABA_A$ receptors (Knoflach et al., 1996; Turner et al., 1991); 2) bretazenil (BTZ), a prototyped non-selective, high-affinity, and low intrinsic efficacy partial positive allosteric modulator (PPAM) of GABA action at several $GABA_A$ receptor subtypes including α1, α2, α3, α4 and α5 subunits (Haefely et al., 1990; Facklam et al., 1992; Jenck et al., 1992; Giusti et al., 1993; Smith et al., 2001).

To this end, we compared the anxiolytic, anticonvulsant action (bicuculline and maximal electroshock seizure tests) and the suppression of locomotor activity mediated by DZ and BTZ to those of IMD and its derivatives [6-(2-bromomophenyl)-N-ethyl-8-fluoro-4H-imidazo-[1,5-a][1,4] benzodiazepine-3-carboxamide (enazenil); 6-(2-bromomophenyl)-8-fluoro-N-(1-methylethyl)-4H-imidazo-[1,5-a][1,4] benzodiazepine-3-carboxamide (RO 25-2775); 6-(2-bromomophenyl)-8-fluoro-N-propyl-4H-imidazo-[1,5-a][1,4] benzodiazepine-3-carboxamide (RO 25-2776), and 6-(2-bromomophenyl)-N-[(cyclo-propyl) methyl]-fluoro-4H-imidazo-[1,5-a][1,4] benzodiazepine-3-carboxamide (RO 25-2847)] in rats and mice (Fig. 1). The overall goal of the study is to determine whether the pharmacological profile of imidazo-benzodiazepine carboxamide derivatives is similar to that of DZ or BTZ.

2. Materials and methods

2.1. Animals

Male Fisher rats weighing 200–250 g and adult male Swiss–Webster mice weighing 25–30 g (Harlan Breeders, Indianapolis) housed in groups of three per cage and maintained on a 13–11-h light/dark cycle (lights from 6:00 am to 7:00 pm) with free access to food and water were used for these studies. Behavioral testing was generally conducted between 10:00 am and 4:00 pm and in accordance with the National Institute of Health, Guide for the Care and Use of Laboratory Animals as approved by the Animal Welfare Committee at the University of Illinois at Chicago.

2.2. Drugs

DZ, BTZ, IMD, enazenil, RO 25-2775, RO 25-2776, and RO 25-2847 were obtained from Hoffman-La Roche (Nutley, NJ); bicuculline (BIC) and pentylenetetrazole were purchased from Sigma-Aldrich Co. (St. Louis, MO); [^{3}H]-flumazenil from NEN (Boston, MA). Pentylenetetrazole was dissolved in saline (0.9% NaCl); DZ, BTZ, IMD, enazenil, RO 25-2775, RO 25-2776, and RO 25-2847 were dissolved in 5–10% of DMSO (depending on the final concentration needed) and subsequently diluted with a vehicle containing 11% polyethylene glycol-400, 50% propylene glycol, and 39% sterile water. The volume of injection for vehicle, DZ, BTZ, IMD, enazenil, RO 25-2775, RO 25-2776, and RO 25-2847 was 1.0 ml/kg body weight for rats and 0.1 ml/10 g for mice.

3. Binding studies

In vitro binding studies were conducted with minor modifications of a previously published method (Massoti et al., 1991). Cerebral cortices obtained from male rats were homogenized in 20 ml ice-cold 0.32 M sucrose by using a glass homogenizer with a Teflon pestle. The homogenate was centrifuged for 10 min at 1000 *g* at 4 °C. The P_1 pellet was discarded and the supernatant was collected and centrifuged at 20,000 *g* at 4 °C for 20 min. The resulting crude mitochondrial pellet (P_2) was resuspended in 20 ml of ice-cold distilled water and homogenized. The homogenate was centrifuged at 8000 *g* at 4 °C for 20 min, the supernatant collected and centrifuged at 48,000 *g* at 4 °C

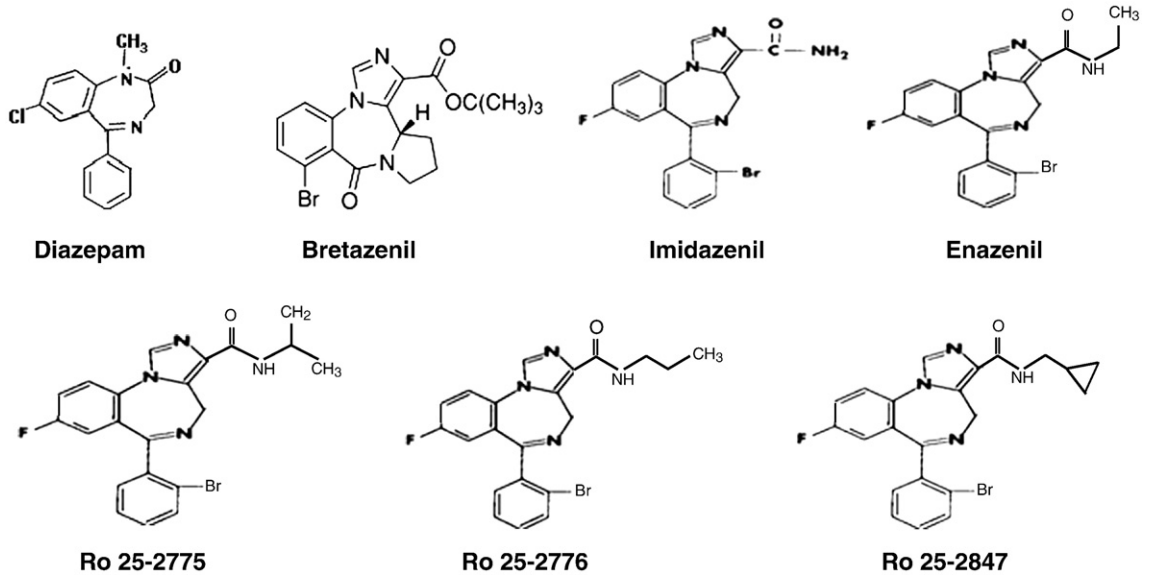

Fig. 1. Structure of diazepam, bretazenil, imidazenil and other imidazo-benzodiazepine carboxamide derivatives (enazenil, Ro 25-2775, Ro 25-2776, Ro 25-2847).

for 20 min and the final crude synaptic membrane pellet (P_3) was stored at −20 °C until used. After thawing, the pellet was resuspended in 10 ml of 50 mM TRIS–HCl, pH 7.4, centrifuged at 48,000 *g* at 4 °C for 20 min and resuspended in 3.5 ml of the same buffer for standard binding assay. Inhibition of [^{3}H]-flumazenil binding by BZ binding site ligands was investigated by incubating 200 μg of membrane protein suspension for 60 min at 4 °C with 1 nM [^{3}H]-flumazenil in the absence or presence of 10 μM of DZ (non-specific binding) or varying concentrations of different BZ ligands in a total volume of 1 ml of 50 mM TRIS–HCl buffer, pH 7.4. All tested compounds were dissolved in DMSO and diluted in the incubation medium immediately before the assay. The maximal DMSO concentration in the final incubation medium was 1% (v/v); this concentration fails to influence the binding of [^{3}H]-flumazenil to brain membranes. The samples were filtered under vacuum through Whatman GF/B filters and washed three times with 5 ml of cold buffer. The competition experiments were run in triplicate with six different concentrations of competing BZ ligands. IC50 ± SEM values for the displacement of [^{3}H]-flumazenil were determined by a nonlinear curve-fitting program based on LIGAND (Munson and Robard, 1980). Statistical comparisons of the estimated parameters were performed using Student's t-test for unpaired samples or analysis of variance followed by Dunnett's test. In all cases, $p<0.01$ was considered statistically significant.

3.1. Anticonvulsant action

3.1.1. Bicuculline seizure test

A 2.7 μmol (1 mg/ml) stock solution of bicuculline HCl was prepared by dissolving (+)-bicuculline in 0.1 N HCl and then diluted with isotonic saline (0.9% NaCl) solution to a final concentration of 0.27 μmol/ml (0.1 mg/ml).The convulsive threshold dose of BIC was determined by infusing 0.27 μmol/ml of BIC into the tail vein of unrestrained and freely moving rats at a constant rate of 0.46 ml/min using a Kd Scientific infusion pump (Model 200, New Hope, PA). Rats received intraperitoneal (i.p.) injections of vehicle or increasing doses of tests compounds 10 min before the start of BIC infusion. The infusion was stopped at the appearance of the first visual sign of tonic–clonic convulsions and the infusion time to elicit tonic–clonic convulsions was recorded. The convulsive threshold dose (expressed in μmol/kg) of bicuculline for each animal was calculated using the time to elicit tonic–clonic convulsions, the infusion rate, and bicuculline concentration (0.27 μmol/ml). The mean (±SEM) threshold dose of bicuculline needed to elicit tonic–clonic convulsions was calculated for each group of rats.

These graded dose–response curves were analyzed (curve fit, sigmoid dose–response with variable) using nonlinear regression with GraphPad Prism (version 4.03, GraphPad Software, San Diego, CA) to estimate anti-BIC ED_{50} (dose that elicits 50% of the maximal effect) values for the respective BZ receptor ligands studied.

3.1.2. Maximal electroshock seizure (MES) test

Tonic hind limb extension was produced in BALB/c mice (weight 25–30 g) using ear-clip electrodes delivering a 48 mA alternating current (0.4 s duration; 50 Hz; 0.4 ms square-wave pulse) 30 min after oral administration of test compounds. Eight animals were used for each dose of the compounds studied. The relative drug potency (ED_{50} = dose of drug that protects 50% of animals from tonic extension) was derived by probit analysis according to Finney (1971).

3.2. Conflict and proconflict tests

The Vogel-conflict test in both its "conflict" and "proconflict" paradigm is a sensitive test that has been used to verify drugs that positively or negatively modulate $GABA_A$ receptor function (Corda et al., 1983). In these experiments, the punishment behavioral paradigm developed by Vogel et al. (1971) and modified by Giusti et al. (1991) was used. In brief, rats weighing 200–250 g were deprived of water for 72 h and placed in a chamber with a water source. Each rat was allowed to become familiar with a habituation chamber (identical to the testing chamber but without a water source) for 5 min immediately before testing to avoid exploration-induced delayed drinking. After habituation, rats were transferred to the testing chamber (28×20×20 cm with a stainless-steel grid floor). Water was provided with a stainless-steel drinking tube (see Giusti et al., 1991 for more detail) and each rat was allowed the completion of a 10 s licking period before the start of a 3 min test session. In the absence of punishment, rats usually lick the water spout almost without interruption for the first 3 min of exposure (test period), totaling about 50 licking periods each lasting 3 s. Programming for the test session was controlled by a solid-state modular programming apparatus (Atto Instruments, Washington, DC). Rats that failed to start drinking within 5 min after exposure to the test chamber were excluded from the experiments.

The two experimental paradigms used here are referred to as "conflict" and "proconflict." In the conflict paradigm, punishment was set at a current intensity of 0.8 mA for 1 s duration. When a current of 0.8 mA is used, the drinking behavior of the rats is virtually suppressed. The number of licking periods (each period equal to 3 s of cumulative drinking) was recorded in unpunished rats and in rats punished with an electric shock (0.8 mA, 1 s duration) delivered through the drinking tube after each drinking period. In contrast, in the proconflict paradigm the intensity of the electric shock delivered as an aversive stimulus was decreased from 0.8 mA to 0.35 mA for 1 s, but in addition rats received a sub-convulsive dose of PTZ (145 mmol/kg i.p.) 15 min before the test. Under the proconflict conditions, PTZ enhances shock-induced suppression of drinking (for details see Giusti et al., 1991). These experimental paradigms were used to study the dose-dependent anticonflict or proconflict action of DZ, BTZ, and IMD and its derivatives (enazenil, RO 25-2775, RO 25-2776, and RO 25-2847). Thus, the terms anticonflict and anti-proconflict are used to denote the protective action of the respective BZ receptor ligands against conflict or proconflict paradigms, respectively.

The anticonflict effects of the respective drugs tested were calculated as follows:

$$Protection = (A-K/B-K) \times 100\ \%$$

where A is the average number of licking periods in a group of drug-treated rats receiving punishment; B is the average number of licking periods in a group of drug-treated rats not receiving punishment; and K is the average number of licking periods in a group of vehicle-treated rats receiving punishment. In the absence of punishment (B), the average number of licking periods observed in drug-treated rats did not differ significantly from those in vehicle-treated rats at the dose tested.

The relative potency (ED_{50} = dose of drug that gives 50% protection) in the conflict and proconflict tests were derived from the percentage of protection data by probit analysis according to Finney (1971). In all these parameters, the fiducial limits were referred to $p=0.01$.

DZ, BTZ, IMD, enazenil, RO 25-2775, RO 25-2776, and RO 25-2847 were injected i.p. 10 min before the test; 10 rats (5 for the conflict and 5 for the proconflict procedure) were used for each dose of the drugs tested.

3.3. Locomotor activity

Locomotor activity was studied in rats and mice using a computerized AccuScan 4 animal activity monitoring activity system (Columbus Instruments) assisted by Versamax software (AccuScan Instruments). Each activity cage consisted of a Perspex box

(20×20×20 cm) surrounded by vertical and horizontal infrared sensor beams. With the aid of the Versamax Windows-based software, the motility of each rat was recorded as the number of horizontal beam interruptions and the total distance traveled. Locomotor activity was recorded between 1:00 pm and 3:00 pm in the animal facility where the mice are housed. Groups of six animals received i.p. injections of increasing doses of DZ, IMD or the anti-BIC ED_{50} dose for BTZ, enazenil, RO 25-2775, RO 25-2776, and RO 25-2847 or vehicle 10 min prior to a 10 min test session.

4. Results

4.1. In vitro binding

Table 1 shows a comparison of the potencies of DZ and BTZ with those of IMD and its derivatives to displace the binding of [^{3}H]-flumazenil from crude cortical synaptic membrane preparation. The IC_{50} values for [^{3}H]-flumazenil displacement indicate that all the compounds tested were more potent than DZ in displacing [^{3}H]-flumazenil from its binding sites. In addition, the potency of RO 25-2775 and IMD was greater than that of BTZ, which was similar to that of RO 25-2776 and RO 25-2847. A comparison of the potencies of IMD and its derivatives to displace [^{3}H]-flumazenil binding shows that RO 25-2775 is the most potent while enazenil is the least potent; the order of potency for these imidazo-benzodiazepine carboxamide derivatives is RO 25-2775>IMD>RO 25-2776≥RO 255-2847>enazenil. These [^{3}H]-flumazenil displacement data indicate that compared to DZ and BTZ, IMD and its derivatives have a higher affinity for the BZ recognition site on the $GABA_A$ receptor complex.

4.2. Potency of positive allosteric modulators of $GABA_A$ receptors in inhibiting BIC or MES-induced seizures and for anticonflict and anti-proconflict effects

Anticonvulsant efficacy of test compounds was evaluated using a bicuculline seizure test in rats and a maximal electroshock seizure test in mice. Fig. 2 shows dose-dependent shifts in the convulsive threshold dose of BIC to induce tonic–clonic convulsions following a 10 min pretreatment with respective BZ receptor ligands. The data shows that all the positive allosteric modulators of GABA action studied resulted in a dose-dependent increase in the convulsive threshold dose of BIC. However, the various BZs studied show different efficacies and potencies against BIC-induced tonic–clonic seizures. As shown in Fig. 2, the anti-BIC actions of the standard BZ studied indicate that while BTZ is not a good anticonvulsant, IMD and DZ are efficacious against BIC-induced tonic–clonic convulsions. Interestingly, three IMD derivatives (enazenil, RO 25-2775, and RO 25-2847) show comparable efficacy to IMD while RO 25-2776 was comparatively less efficacious than IMD, enazenil, RO 25-2847, and RO 25-2775. Thus, as shown in Fig. 2, the rank order for the anti-BIC efficacy for these BZ receptor ligands is DZ>IMD≥RO 25-2775≥RO 25-2847>enazenil>RO 25-2776>BTZ (Fig. 2).

Table 1
IC_{50} for inhibition of [^{3}H]-flumazenil binding to rat cortical membranes by different imidazo-benzodiazepines, diazepam, and bretazenil.

DRUGS	IC_{50} nM
Imidazo-benzodiazepines	
Imidazenil	0.92±0.08
Enazenil	5.50±0.20
RO 25-2775	0.46±0.10
RO 25-2776	1.70±0.09
RO 25-2847	1.90±0.19
Others	
Diazepam	48.0±3.0
Bretazenil	2.60±0.36

The IC_{50} values were calculated using a non linear curve-fitting program based on LIGAND (Munson and Robard, 1980).

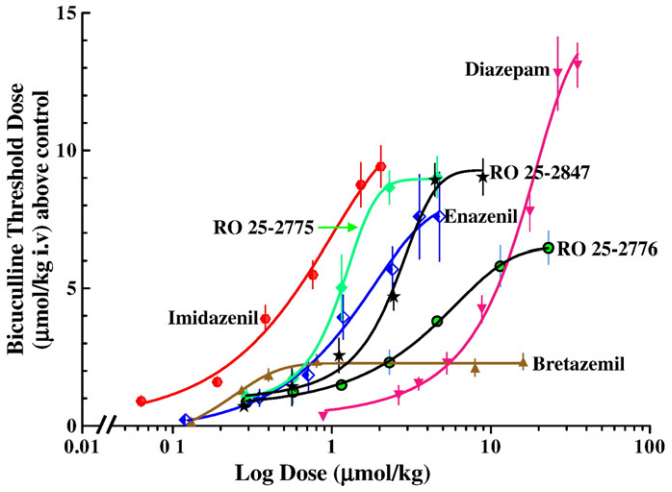

Fig. 2. Dose-dependent increases in bicuculline (BIC) tonic–clonic convulsion threshold by various positive allosteric modulators of $GABA_A$ receptors. Animals received intraperitoneal (i.p.) injections of vehicle or increasing doses of the various anticonvulsant BZ receptor ligands 10 min prior to bicuculline (0.27 μmol/ml) infusion. The threshold dose of BIC needed to induce convulsions in vehicle-treated rats (1.41±0.2 μmol/kg i.v.) was subtracted from the dose of BIC required to induce convulsions in rats pretreated with different doses of the respective anticonvulsant benzodiazepines. Each value is the mean±S.E.M. of 4 to 6 rats.

A comparison of the *in vivo* potency of DZ and BTZ to that of IMD and its derivatives to shift the threshold of bicuculline and electrically-induced tonic–clonic convulsions in rats and mice, respectively, is shown in Table 2. In addition, we also studied the potency of these compounds to elicit anticonflict and anti-proconflict effects in rats. Note that the potency of BTZ was evaluated in all behavioral paradigms except in MES. Table 2 shows the respective ED_{50} values of the different BZ ligands for each of the behavioral paradigms studied.

A comparison of the anti-BIC potency of these positive allosteric modulators of GABA action indicates that IMD is the most potent. Except for BTZ, which was virtually inactive, all the compounds tested were more potent than DZ. Although all four IMD derivatives studied were less potent than IMD, they were 4–18-fold more potent than DZ against BIC-induced tonic–clonic convulsions. Thus, the rank order for the anti-BIC potency for these BZ receptor ligands is IMD>RO-25-2775>enazenil>RO-25-2847>RO-25-2776>DZ. The results for

Table 2
Potency of positive allosteric modulators of $GABA_A$ receptors for inhibiting BIC or MES-induced seizures, anticonflict, and anti-proconflict effects.

DRUG	ED_{50} (μmol/kg)			
	Anti-bicuculline	Anti-MES	Anticonflict effect	Anti-proconflict effect
Diazepam	20.4	0.47 (0.31–0.71)	2.0 (1.4–2.8)	1.90 (1.5–2.5)
Bretazenil	ND	NT	2.40 (1.7–3.1)	0.20* (0.12–0.43)
Imidazenil	0.91	0.15* (0.10–0.22)	2.90 (1.0–5.9)	0.061* (0.024–0.22)
Enazenil	1.55	0.32 (0.18–0.56)	2.80 (1.2–6.3)	0.079* (0.051–0.13)
Ro 25-2775	1.16	0.48 (0.24–1.00)	1.72 (0.45–6.4)	0.52* (0.09–2.90)
Ro 25-2776	4.81	0.34 (0.19–0.62)	2.74 (1.6–4.8)	0.44* (0.12–1.5)
Ro 25-2847	2.57	0.16* (0.10–0.27)	3.30 (2.1–5.4)	0.43* (0.10–1.8)

The ED_{50} values and fiducial limits (*$p<0.05$; in parenthesis) were calculated from the percent protection data by probit analysis according to Finney (1971); *$p<0.01$ when compared to diazepam. ND = not determined; NT = not tested.

protection against electrically-induced seizures indicate that IMD and one of its derivatives (RO 25-2847) were 3-fold more potent than DZ whereas the other three IMD derivatives, RO 25-2775, RO 25-2776 and enazenil were equipotent to DZ (Table 2).

We have previously shown that the shock-induced suppression of water drinking (conflict paradigm) and the pentylenetetrazole-enhanced suppression of water drinking in thirsty rats (proconflict paradigm) can be used as animal models to test the anxiolytic and antipanic actions of positive allosteric modulators of GABA action, respectively (Giusti et al., 1991).Table 2 shows the ED_{50} values for the anticonflict and anti-proconflict actions of the BZ receptor ligands tested. These data show that all the compounds tested elicit anticonflict (anxiolytic action) and anti-proconflict (antipanic action) effects. Although the anticonflict ED_{50} values for DZ, BTZ, IMD, enazenil, and RO 25-2776 are similar, their anti-proconflict ED_{50} values are much lower than their respective anticonflict ED50 values. For example, the ED_{50} values for the anti-proconflict action of DZ, BTZ, IMD, enazenil, and RO 25-2776 are 0.2 and 0.061, 0.079, and 0.44 respectively. It is important to note that unlike DZ, which shows a similar potency in both tests, IMD and its derivatives are more potent in the proconflict than in the conflict test. In addition, the ED_{50} values also show that in the proconflict test, IMD is the most potent drug tested whereas DZ is the least potent. However, in the conflict test the ED_{50} values show that RO 25-2775, the IMD derivative with a small carboxamide alkyl substitution, is the most potent while RO 25-2847, which has the a long carboxamide alkyl substitution, is the least potent.

4.3. Suppression of locomotor activity

To evaluate the sedative effects of these positive allosteric modulators of GABA action, we studied the effects of these drugs on the suppression of locomotor activity using three doses of DZ, IM or the anti-BIC ED_{50} doses for RO-25-2847, RO-25-2775, RO-25-2776, BTZ, or enazenil. The results indicate that that there are highly significant differences among groups [$p<0.0001$ for both horizontal activity and total path length]. Specifically, IMD administered in doses 3, 16, and 42-fold higher than the ED_{50} for anti-BIC, anti-MES, and antipanic actions respectively had no appreciable effect on horizontal activity counts of total path length traveled (Fig. 3A and B). In contrast, DZ administered in doses 4-fold higher than the ED_{50} doses for antipanic and anxiolytic actions or 2-fold lower than the ED_{50} for anti-BIC action remarkably suppressed both horizontal activity counts and total path length (Fig. 3A and B) traveled. In Fig. 3C and D we also demonstrate that the anti-BIC ED_{50} for RO-25-2847, RO-25-2776, BTZ, and enazenil all significantly suppressed horizontal activity counts and total path length traveled. Like IMD and in contrast to the other imidazo-benzodiazepine carboxamide derivatives, the anti-BIC ED_{50} dose for RO-25-2775 slightly suppressed horizontal activity but had no significant effect on total path length traveled. It is interesting to note that when compared to the vehicle-treated group, DZ administered in a dose less than one-half the anti-BIC ED_{50} dose resulted in a remarkable (90% and 87% decrease in horizontal counts and total path length traveled respectively) suppression of locomotor activity. Similar results on locomotor activity were obtained in mice (data not shown).

5. Discussion

The therapeutic application of DZ and other high efficacy and non-selective BZ (e.g., triazolam, alprazolam, midazolam, flunitrazepam etc.) receptor ligands that potentiate GABA action at a wide spectrum of $GABA_A$ receptor subtypes have been associated with a number of unwanted effects such sedation, amnesia, ataxia, and high tolerance and dependence liabilities. The high-affinity binding and ultimately maximal efficacy of BZ-mediated positive allosteric modulation of GABA action at $GABA_A$ receptors are conferred by the expression of $\alpha1$, $\alpha2$, $\alpha3$, or $\alpha5$ subunits and the presence of an adjacent $\gamma2$ subunit (Pritchett et al., 1989; Pritchett and Seeburg, 1990).

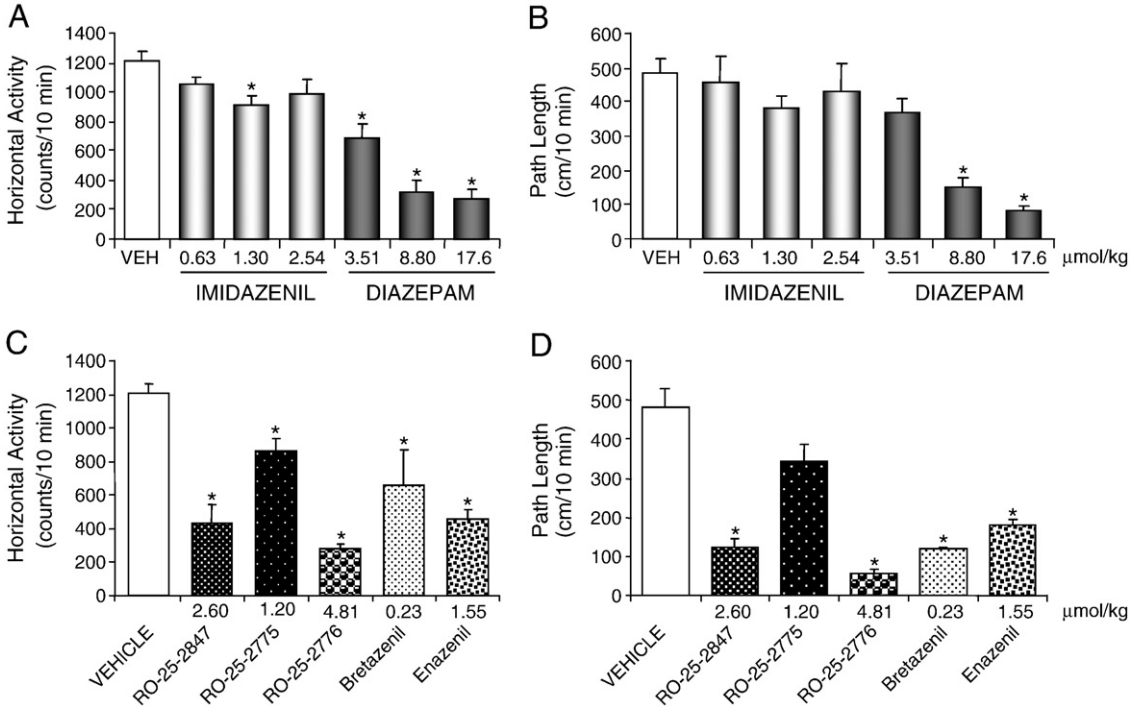

Fig. 3. Dose-dependent effects of imidazenil (IMD), diazepam (DZ) [A and B] or the ED_{50} anti-bicuculline for bretazenil (BTZ), enazenil, RO 25-2775, RO 25-2776, and RO 25-2847 (C and D) on locomotor activity [horizontal activity (A andC) and path length traveled (B and D)] in rats. Groups of rats received intraperitoneal (i.p.) injections of vehicle, increasing doses of IMD, DZ or the ED_{50} anti-bicuculline dose for BTZ, RO 25-2775, RO 25-2776, and RO 25-2847 10 min prior to a 10 min test session. Each bar is the mean ± SEM of 6 rats per group. Data were subjected to ANOVA followed by the Duncan multiple comparison test. *$p<0.05$ compared to vehicle treatment.

Advances in gene-targeted approaches have yielded valuable information on the specific roles of selective $GABA_A$ receptor subunits in the pharmacology of positive allosteric modulators of GABA action at $GABA_A$ receptors. For example, several studies have demonstrated that α1-containing $GABA_A$ receptors mediate the sedative–hypnotic and amnestic actions (Rudolph et al., 1999; McKernan et al., 2000; Low et al., 2000; Crestani et al., 2000; Kralic et al., 2002; Rowlett et al., 2005) while α2-containing $GABA_A$ receptors mediate the anxiolytic effects (Low et al., 2000; Mckernan et al., 2000; Rowlett et al., 2005; Whiting, 2006) of non-selective BZ receptor ligands such as DZ. In addition, tolerance liability has also been associated with the protracted amplification of GABA action at α1-containing $GABA_A$ receptor subtypes (Crestani et al., 2001; Mohler et al., 2001).

In searching for more selective, efficacious and safer BZ receptor ligands, we have been investigating the pharmacology of imidazo-benzodiazepine carboxylic acid BZ recognition site ligands. In this regard, we have extensively studied the pharmacology of IMD, a prototypic imidazo-benzodiazepine carboxylic acid, in rodents and non-human primates. In brief, we have demonstrated that IMD has low intrinsic efficacy at α1- but high intrinsic efficacy at α5-containing $GABA_A$ receptors (Guidotti et al., 2005). Most importantly, IMD elicits potent anticonvulsant and anxiolytic actions at doses that are virtually devoid of sedative and amnestic effects (Giusti et al., 1993; Auta et al., 1994, 1995, 2000, 2004; Costa et al., 2001, 2002; Impagnatiello et al., 1996; Guidotti et al., 2005). In this report we compared the pharmacological profile of IMD and its derivatives to that of DZ and BTZ.

The results of our binding study show that like DZ and BTZ, IMD and its derivatives displaced [^{3}H]-flumazenil binding with nanomolar affinity. It is important to note that the affinity of IMD and its derivatives for cortical [^{3}H]-flumazenil binding sites is similar to that of BTZ but much higher than that of DZ. To evaluate whether the high-affinity binding of IMD and derivatives to [^{3}H]-flumazenil recognition sites is associated with any pharmacological effects, we evaluated the behavioral effects associated with the administration of these compounds using several behavioral paradigms. Table 2 is a comparative summary of the potency of IMD and derivatives, DZ, and BTZ in eliciting various behavioral actions that are mediated by a positive allosteric modulation of GABA action by these BZ receptors ligands at $GABA_A$ receptor subtypes. In this table we show that DZ, the non-selective FPAM of GABA action, suppresses locomotor activity (see Fig. 3) and has similar ED_{50} values for anticonflict and anti-proconflict actions with a significantly higher ED_{50} value for anti-BIC action and a much lower ED_{50} value for anti-MES. Similar to DZ, BTZ also suppresses locomotor activity; however, its anticonflict ED_{50} value is significantly higher than that for anti-proconflict effect while exhibiting minimal anti-BIC action. In contrast to DZ, IMD (SPAM) has significantly lower ED_{50} values for anti-proconflict and anti-BIC action with a significantly higher ED_{50} value for anticonflict action but does not suppress locomotor activity. A comparison of the potency for anticonflict, anti-proconflict, anti-BIC, anti-MES and locomotor suppressive effects of these compounds shows that IMD and its derivative, RO-25-2775, elicit potent anxiolytic, antipanic, and anticonvulsant effects in rats but failed to suppress locomotor activity in rats and mice. Although other derivatives (RO25-2776 and RO 25-2847) of IMD also elicit anxiolytic, antipanic, and anticonvulsant effects, these actions were associated with significant suppression of locomotor activity. Furthermore, we demonstrate here that IMD and its derivatives have good specificity in their separation index because the anticonflict, anti-BIC, and anti-MES ED_{50} values are much higher than the anti-proconflict ED_{50} values. In contrast, the anticonflict and anti-proconflict ED_{50} values for DZ are similar and are associated with suppression of locomotor activity, suggesting that DZ does not have an adequate separation index.

Based on the current understanding of the role of various $GABA_A$ receptor subunits on the pharmacology of BZ-mediated amplification of selective $GABA_A$ receptor subtypes, one can infer that the profound suppression of locomotor activity elicited by DZ and two IMD derivatives might be due to their high intrinsic efficacy at α1-containing $GABA_A$ receptors (Crestani et al., 2001; Mohler et al., 2001; Guidotti et al., 2005). Furthermore, we have previously shown that at doses that produce anxiolytic and anticonvulsant actions, IMD antagonized the sedative and amnestic actions of alprazolam in both rodents and primates (Auta et al., 2000, 2005; Costa et al., 2001). The anxiolytic, anti-proconflict and in part the anticonvulsant actions of IMD and its derivatives are probably mediated by their high intrinsic efficacy on α2, α3, or α5-containing $GABA_A$ receptors and lack of intrinsic activity at α1-containing $GABA_A$ receptors (Guidotti et al., 2005; Costa et al., 2002).

Our data also suggest that the length of the alkyl substitution at the carboxamide group of IMD most likely results in derivatives with increased intrinsic efficacy for α1-containing $GABA_A$ receptor subtypes because RO 25-2776 and RO 25-2847, which have longer carboxamide alkyl substitutions, produced significant suppression of locomotor activity. Thus, these IMD derivatives might elicit pharmacological actions that are similar to that of DZ and other FPAMs. In contrast, RO 25-2776, which has a shorter carboxamide alkyl substitution, elicits potent anticonvulsant action but fails to produce significant suppression of locomotor activity. This suggests that this imidazo-benzodiazepine carboxylic acid derivative might have a pharmacological profile similar to that of IMD.

We have previously shown that the clearance rate for IMD is slower (in rats T1/2 is 180 min; and in non-human primates it is >8 h) than that for DZ (Giusti et al., 1993; Costa and Guidotti, 1996). However, we do not know whether these IMD structural modifications resulted in derivatives with a decreased or increased disposition rate. Furthermore, the time course for IMD derivative action needs to be investigated.

In summary, IMD and two of its carboxamide derivatives possess dose-dependent anticonflict, anti-proconflict, and anticonvulsant (anti-bicuculline and anti-MES) actions but failed to induce sedation, as indicated by their failure to suppress locomotor activity in rats and mice. Whereas other IMD derivatives (RO 25-2776 and RO 25-2847) elicit behavioral actions that are similar to those of DZ, no imidazo-benzodiazepine derivative shares a similar pharmacological profile with BTZ. Although DZ possesses dose-dependent anticonflict, anti-proconflict, and anticonvulsant actions, it also produced sedation, as indicated by the marked suppression of locomotor activity we found. Finally, we have also shown that while DZ does not have a separation index, IMD and two of its derivatives have a good separation index among their different pharmacological actions. However, the pharmacokinetic profile and duration of action of the two IMD derivatives that show a similar pharmacological profile to IMD need further investigation.

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