Characterization of COR627 and COR628, Two Novel Positive Allosteric Modulators of the GABA<sub>B</sub> Receptor

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ABSTRACT

The potential efficacy of GABA<sub>B</sub> receptor agonists in the treatment of pain, drug addiction, epilepsy, cognitive dysfunctions, and anxiety disorders is supported by extensive preclinical and clinical evidence. However, the numerous side effects produced by the GABA<sub>B</sub> receptor agonist baclofen considerably limit the therapeutic use of this compound. The identification of positive allosteric modulators (PAMs) of the GABA<sub>B</sub> receptor may constitute a novel approach in the pharmacological manipulation of the GABA<sub>B</sub> receptor, leading to fewer side effects. The present study reports the identification of two novel compounds, methyl 2-(1-adamantanecarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate (COR627) and methyl 2-(cyclohexanecarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate (COR628), which act as GABA<sub>B</sub> PAMs in 1) rat cortical membranes and 2) in vivo assay. Both compounds potentiated GABA- and baclofen-stimulated guanosine 5'-O-(3-[35S]-thio)-triphosphate binding to native GABA<sub>B</sub> receptors, while producing no effect when given alone. GABA concentration-response curves in the presence of fixed concentrations of COR627 and COR628 revealed an increase of potency of GABA rather than its maximal efficacy. In radioligand binding experiments [displacement of the GABA<sub>B</sub> receptor antagonist, 3-N-[1-(S)-3,4-dichlorophenyl]-ethylaminol]-2-(S)hydroxypropyl cyclo-hexylmethyl phosphinic acid ([3H]CGP54626), both COR627 and COR628 increased the affinity of high- and low-affinity binding sites for GABA, producing no effect when administered alone up to a concentration of 1 mM. In vivo experiments indicated that pretreatment with per se ineffective doses of COR627 and COR628 potentiated the sedative/hypnotic effect of baclofen. In conclusion, COR627 and COR628 may represent two additional tools for use in investigating the roles and functions of positive allosteric modulatory binding sites of the GABA<sub>B</sub> receptor.

Introduction

GABA, a major inhibitory neurotransmitter present in the central nervous system, exerts its action on two receptor classes, ionotropic GABA<sub>A</sub> and GABA<sub>C</sub>, and metabotropic GABA<sub>B</sub> receptors (Bovery, 2010). The GABA<sub>B</sub> receptor is a member of the “family C” G protein-coupled receptors (GPCRs) (Couvé et al., 2000), including also metabotropic glutamate (mGlu) receptors 1 to 8, Ca<sup>2+</sup>-sensing receptors, vromeronasal, pheromone, and putative taste receptors (Pin et al., 2003). GABA<sub>B</sub> receptors are heteromeric structures composed of two types of subunits, GABAB<sub>H1a,b</sub> and GABAB<sub>H2</sub>, which share structural homology with other family C GPCRs and contain topologically distinct domains: a long extracellular amino-terminal domain, which exhibits a Venus flytrap domain segment, and the intracellular carboxyl domain involved in receptor activation and G-protein coupling (Kauppinen et al., 2003). GABA<sub>B</sub> receptors couple to G<sub>α</sub> protein and activate PLC and IP<sub>3</sub> pathways, leading to the hydrolysis of membrane-associated phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-triphosphate, which triggers an increase in intracellular calcium release.

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The mechanism of agonist-induced receptor activation of GABA<sub>B1</sub> and GABA<sub>B2</sub> heterodimers is unique among GPCRs. In the heteromer, GABA and all other receptor agonists and antagonists bind only to the Venus flytrap of the GABA<sub>B1</sub> (<sub>b</sub>) subunit (Galvez et al., 2000), whereas the GABA<sub>B2</sub> subunit is required for cell membrane expression, effective G-protein coupling and activation (Galvez et al., 2001), and high-affinity binding of agonists to GABA<sub>B1</sub> (Galvez et al., 2001).

GABA<sub>B2</sub> receptors located in cerebellum, thalamus, striatum, cortex, and spinal cord are probably implicated in numerous physiological and pathophysiological conditions, including pain, drug addiction, epilepsy, cognitive function, anxiety, and depression (Bettler et al., 2004). To date, preclinical and clinical studies have evaluated the potential efficacy of GABA<sub>B2</sub> receptor agonists and antagonists in the pharmacological treatment of anxiety and mood disorders, cognitive dysfunctions, and alcohol, cocaine, heroin, and nicotine addiction (Froestl, 2011). As an example, the prototypical GABA<sub>B2</sub> receptor agonist, baclofen, decreased 1) self-administration of alcohol, cocaine, heroin, and nicotine in laboratory rodents and 2) intake of alcohol, cocaine, and nicotine in humans (Vlachou and Markou, 2010). This compound has also displayed anxiolytic properties in clinical and preclinical investigations (Cryan and Kaupsnn, 2005). Baclofen, first introduced into clinical practice as an antiarrhythmic agent more than 30 years ago, is the only marketed drug to act via GABA<sub>B2</sub> receptors (Froestl, 2011). However, its poor blood-brain-barrier penetration, short duration of action, rapid tolerance, and narrow therapeutic window (because of the occurrence of sedative effects at doses close to those producing the “desired” effects) (Vacher and Bettler, 2003) greatly limit its clinical use.

Positive allosteric modulation is a recently identified phenomenon providing novel tools for the pharmacological manipulation of GPCRs; these compounds act at a site apart from the orthosteric binding region of the receptor protein (see Urwyler, 2011). In recent years, several highly selective positive allosteric modulators (PAMs) have been identified for family C GPCRs, including Ca<sup>2+</sup>-sensing receptors, mGlur receptors (Urwyler, 2011), and GABA<sub>B2</sub> receptors (GABA<sub>B2</sub> PAMs) (Urwyler et al., 2001, 2003; Guery et al., 2007; Malherbe et al., 2008). PAMs display no or minimal agonist activity, but enhance both potency and efficacy of orthosteric agonists, and produce fewer side effects and less tolerance than orthosteric agonists (Gjonv and Urwyler, 2008; Urwyler, 2011). 3.5-Bis(1,1-dimethylthyl)-4-hydroxy-β,β-dimethyl-benzene propanol (CGP7930), N,N'-Dicyclopropylent-2-(methylthio)-5-nitro-4,6-pyrimidinediamine (GS39783), N-(1R,2R,4S)-biclo[2.2.1]hept-2-yl-2-methyl-5-[4-(trifluoromethyl)phenyl]-4-pyrimidinediamine (BHF177), and (R,S)-5,7-di-tert-butyl-3-hydroxy-3-trifluoromethyl-3H-benzo-furan-2-one (rac-BHFP), the most widely characterized GABA<sub>B2</sub> PAMs, have been found to increase GABA potency and efficacy at both recombinant and native GABA<sub>B2</sub> receptors in various in vitro assays (Urwyler et al., 2001, 2003; Olianas et al., 2005; Malherbe et al., 2008) and in vivo procedures (Carai et al., 2004; Gjonv et al., 2006; Malherbe et al., 2008; Koek et al., 2010). GABA<sub>B2</sub> PAMs display anxiolytic (Cryan et al., 2004; Frankowska et al., 2007) and antidepressant (Frankowska et al., 2007) properties and reduce self-administration of alcohol and different other behaviors motivated by alcohol (Liang et al., 2006; Maccioni et al., 2009, 2010), cocaine (Smith et al., 2004; Filip et al., 2007), and nicotine (Mombereau et al., 2007; Paterson et al., 2008) in rats. In light of the considerable therapeutic potential of GABA<sub>B2</sub> PAMs, the present study, using [3H]3-N-[(S)-3,4-dichlorophenyl]-ethylaminol-2-(S)-hydroxypropyl cyclo-hexymethyl phosphinic acid ([3H]CGP54626) binding and a widely used functional assay for GPCRs, 5′-O-(3-[(35S)thiatriphosphate]) ([35S]GTPγS) binding, reports the characterization of two novel GABA<sub>B2</sub> PAMs: methyl 2-(1-adamantanecarbox-amido)-4-ethyl-5-methylthiophene-3-carboxylate (COR627) and methyl 2-(cyclohexanecarboxamido)-4-ethyl-5-methylthiophene-3-carboxylate (COR628). These compounds were identified by means of a virtual screening protocol and produced by chemical synthesis in house. The ability of COR627 and COR628 to potentiate baclofen-induced but not pentobarbital-induced loss of righting reflex (LORR) (a valid test to assess the in vivo potential of putative GABA<sub>B2</sub> PAMs (Carai et al., 2004; Malherbe et al., 2008; Koek et al., 2010) was also evaluated.

**Materials and Methods**

All experimental procedures used in the present study were conducted in accordance with the Italian law on the “protection of animals used for experimental and other scientific reasons.”

**Drugs**

[35S]GTPγS and [3H]CGP54626 were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA) and American Radiolabeled Chemicals (St. Louis, MO). GABA, GDP, GTPγS, (R)-4-amino-3-(4-chlorophenyl)butanoic acid [R-(-)-baclofen], (5α,6α)-7,8-dihydroxy-4,5-epoxy-17-ethyl-3,6-diol (morphine), and l-glutamate were obtained from Sigma/RBI (Natick, MA; CGP54626 was obtained from Torcia Bioscience (Ellisville, MO). COR627 and COR628 were synthesized as reported below.

**Chemical Synthesis**

COR627. A solution of 2-amino-4-ethyl-5-methylthiophene-3-carboxylic acid methyl ester (Sabnis et al., 1999) (65 mg, 0.33 mmol), triethylamine (68 μl, 0.5 mmol), and 1-adamantanecarboxyl chloride (97 mg, 0.5 mmol) in dry dichloromethane (5 ml) was kept at room temperature overnight and then poured into saturated sodium bicarbonate solution (10 ml). The organic layer was separated, washed successively with 1 N HCl and brine and subsequently dried over anhydrous sodium sulfate and evaporated under reduced pressure. The crude residue was purified by flash chromatography on silica gel, eluting with hexanes-diethyl ether (8/1). The appropriate fractions were further purified by preparative thin-layer chromatography (silica gel/hexanes-diethyl ether, 6:1) to yield COR627 as a colorless solid (116 mg, 98%). 1H NMR (400 MHz, CDCl<sub>3</sub>): δ 11.49 (s, 1H), 3.83 (s, 3H), 2.65 (q, J = 8.0 Hz, 2H), 2.18 (s, 3H), 2.03 (br s, 3H), 1.91 (br s, 6H), 1.69 (br s, 6H), 0.98 (t, J = 8.0 Hz, 3H); liquid chromatography/mass spectrometry (m/z) = 362 (M + H) (Fig. 1A).

COR628. A solution of 2-amino-4-ethyl-5-methylthiophene-3-carboxylic acid methyl ester (65 mg, 0.33 mmol), triethylamine (68 μl, 0.5 mmol), and cyclohexanecarboxyl chloride (67 μl, 0.5 mmol) in dry dichloromethane (5 ml) was kept at room temperature overnight and then was poured into saturated sodium bicarbonate solution (10 ml). The organic layer was separated, washed successively with 1 N HCl and brine, and subsequently dried over anhydrous sodium sulfate and evaporated under reduced pressure. The crude residue was purified by flash chromatography on silica gel, eluting with hexanes-diethyl ether (4:1) to yield COR628 as a colorless solid (94 mg, 93%). 1H NMR (400 MHz, CDCl<sub>3</sub>): δ 11.30 (s, 1H), 3.81 (s, 3H), 2.65 (q, J = 7.5 Hz, 2H), 2.30 (m, 1H), 2.18 (s, 3H), 1.94 (m, 2H), 1.76 (m, 2H), 1.63 (m, 2H), 1.46 (m, 2H), 1.29 (m, 2H), 0.98 (t, J = 7.5 Hz, 3H); liquid chromatography/mass spectrometry (m/z) = 310 (M + H) (Fig. 1A).
at 20,000 g layer on the pellet was then centrifuged at 45,000 g of ice-cold water, homogenized using a Polytron homogenizer, and centrifuged at 45,000 g. The resulting pellet was resuspended in ice-cold distilled water and once more centrifuged at 45,000 g. The supernatant together with the buffy coat was then centrifuged at 48,000 g for 15 min, and centrifugation was continued three cycles of washing, incubation at 22°C for 15 min, and centrifugation at 48,000 g for 15 min at 4°C. [3H]GCP54626 binding was performed as described previously (Pibiri et al., 2005) using 50 μg of membrane proteins, 2 nM [3H]GCP54626 in a volume of 1 ml at 22–24°C for 30 min. Nonspecific binding was estimated in the presence of 10 μM unlabeled GCP54626. Free ligand was separated from bound ligand by rapid filtration through Whatmann GF/B glass filters using a Brandel 30-sample harvester (Brandel Inc., Gaithersburg, MD). Filters were then rinsed twice with ice-cold Krebs-Henseleit buffer. Filter-bound radioactivity was counted in a liquid scintillation counter (Tri-Carb 1600; PerkinElmer Life and Analytical Sciences) using 3 ml of scintillation fluid (Ultima Gold MV; PerkinElmer Life and Analytical Sciences).

[3H]GCP54626 displacement curves were determined using serial dilutions ranging from 10⁻⁹ to 5 × 10⁻³ M GABA, COR627, or COR628. For modulation experiments, GABA_B agonist (GABA_B) was incubated in assay buffer (50 mM Tris-HCl, 3 mM MgCl₂, and 1 mM EGTA, pH 7.4) at 30°C for 1 h. Independent experiments were repeated on membrane preparations from at least three different brains.

The Bradford (1976) protein assay was used for protein determination using bovine serum albumin as a standard according to the supplier protocol (Bio-Rad, Milan, Italy). The calculation of IC₅₀ was performed by nonlinear curve fitting of the concentration-effect curves using GraphPad Prism (GraphPad Software Inc., San Diego, CA). The F test was used to determine the best approximation of a nonlinear curve fitting to one or two site model (P < 0.05).

[35S]GTPγS binding assay in rat cortical and striatal membranes. For GABA₄ and glutamate-stimulated [35S]GTPγS binding assays, rat cortex membranes were thawed at 4°C, resuspended in 1 to 2 ml of ice-cold water, and homogenized using a homogenizer system (Glass-Col). Membranes were incubated on ice for 1 h and then centrifuged at 4°C for 15 min at 20,000g. The pellet was resuspended in GTPγS buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM MgCl₂, and 1.8 mM CaCl₂) to a final concentration of 10 to 15 μg of protein. Membrane homogenates and drugs were preincubated in PerkinElmer PicoPlates 96 (300-μl volume) in the presence of 30 μM GDP for 30 min at 30°C. The main incubation was subsequently started by the addition of [35S]GTPγS to a final concentration of 0.2 nM. After a 40-min incubation at 30°C, the samples were filtered using a PerkinElmer UniFilter-GF/B, washed twice with 300 μl of buffer, and dried at 90°C for at least 18 h before use both for [3H]GCP54626 binding assay and [35S]GTPγS binding assay for GABA₄ and L-glutamate.

For μ-opioid-stimulated [35S]GTPγS binding, striatal membranes (10–15 μg of protein) were incubated in assay buffer (50 mM Tris-HCl, 3 mM MgCl₂, 0.2 mM EGTA, and 100 mM NaCl, pH 7.4) at 30°C for 1 h with 30 μM GDP and 0.05 nM [35S]GTPγS in a volume of 1 ml. After incubation, the samples were filtered using a PerkinElmer UniFilter-GF/B, washed 2 times with 300 μl of buffer, and dried at 90°C for at least 18 h before use both for [3H]GCP54626 binding assay and [35S]GTPγS binding assay for GABA₄ and L-glutamate.

Animals

Male Sprague-Dawley rats and DBA mice (Charles River Laboratories, Calco, Italy), weighing 200 to 250 and 17 to 20 g, respectively, were used. Rats and mice were housed 4 and 20/cage, respectively, in standard plastic cages with wood chip bedding, under an inverted 12:12-h artificial light/dark cycle (lights on at 9:30 PM) at a constant temperature of 22±2°C and relative humidity of approximately 60%. Tap water and standard laboratory rodent chow (Mucedola, Settimo Milanese, Italy) were provided ad libitum in the home cage.

In Vitro Experiments

Binding Studies. Tissue preparation. Rats were killed by decapitation, their brains were rapidly removed, and cerebral cortices were dissected on ice. Cortical tissue was homogenized using a homogenizer system (Glass-Col, Terre Haute, IN) in 20 volumes (v/w) of ice-cold 0.32 M sucrose containing 1 mM EDTA. The homogenate was centrifuged at 1000g for 10 min, and the supernatant was collected and recentrifuged at 20,000g for 20 min. The pellet was resuspended in 20 volumes (v/w) of ice-cold water, homogenized using a Polytron homogenizer, and centrifuged at 8000g for 20 min. The supernatant together with the buffy layer on the pellet was then centrifuged at 45,000g for 20 min. The resulting pellet was resuspended in ice-cold distilled water and once more centrifuged at 45,000g for 30 min. The final pellet was frozen and stored at −80°C for at least 18 h before use both for [3H]GCP54626 binding assay and [35S]GTPγS binding assay for GABA₄ and L-glutamate.

For μ-opioid-stimulated [35S]GTPγS binding, striata were dissected and homogenized in 20 volumes of buffer (50 mM Tris-HCl, 3 mM MgCl₂, and 1 mM EGTA, pH 7.4). The homogenate was centrifuged twice at 48,000g at 4°C for 10 min, resuspended in homogenization buffer, and frozen until use.

[3H]GCP54626 binding assay. On the experiment day, membrane pellets were thawed at 4°C, resuspended in 20 volumes (v/w) of ice-cold Krebs-Henseleit buffer (143 mM NaCl, 50 mM Tris, 5.9 mM KCl, 1.2 mM MgSO₄, and 2.5 mM CaCl₂, pH 7.4) and then homogenized. To remove GABA_B agonist, endogenous membranes underwent three cycles of washing, incubation at 22°C for 15 min, and centrifugation at 48,000g for 15 min at 4°C. [3H]GCP54626 binding was performed as described previously (Pibiri et al., 2005) using 50 μg of membrane proteins, 2 nM [3H]GCP54626 in a volume of 1 ml at 22–24°C for 30 min. Nonspecific binding was estimated in the presence of 10 μM unlabeled GCP54626. Free ligand was separated from bound ligand by rapid filtration through Whatmann GF/B glass filters using a Brandel 30-sample harvester (Brandel Inc., Gaithersburg, MD). Filters were then rinsed twice with ice-cold Krebs-Henseleit buffer. Filter-bound radioactivity was counted in a liquid scintillation counter (Tri-Carb 1600; PerkinElmer Life and Analytical Sciences) using 3 ml of scintillation fluid (Ultima Gold MV; PerkinElmer Life and Analytical Sciences).
for 1 h at 30°C. The radioactivity on the filters was counted in a liquid microplate scintillation counter (TopCount NXT; PerkinElmer Life and Analytical Sciences) using 50 µl of scintillation fluid (Microscint 20; PerkinElmer Life and Analytical Sciences). Basal binding was assessed in the absence of agonist and in the presence of GDP, and nonspecific binding was measured in the presence of 10 µM unlabeled GTPγS. The stimulation by agonist was defined as a percentage increase above basal levels (i.e., [disintegrations per minute (agonist) – disintegrations per minute (no agonist)]/disintegrations per minute (no agonist)) × 100). Data are reported as means ± S.E.M. of three to six experiments, performed in triplicate. Nonlinear regression analysis of concentration-response data was performed using Prism 2.0 software (GraphPad Prism) to calculate \( E_{\text{max}} \) and \( EC_{50} \) values.

Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Dunnett’s test for post hoc comparisons.

**In Vivo Experiments**

**Baclofen Studies.** Two independent experiments were conducted, one testing COR627 and one testing COR628. COR627 (0, 3, 10, 30, 100, and 300 mg/kg) and COR628 (0, 3, 10, 30, 100, and 300 mg/kg) were administered intraperitoneally 30 min before the injection of a fixed dose of baclofen (35 mg/kg i.p.) to independent groups of \( n = 6 \) mice each. COR627 and COR628 were suspended in saline with a few drops of Tween 80. Baclofen was dissolved in saline. All drugs were injected at a volume of 12.5 ml/kg. The dose ranges of COR627 and COR628 were chosen on the basis of the results of a series of preliminary experiments demonstrating that they were totally devoid of any sedative/hypnotic effect in DBA mice (this laboratory, unpublished results). The baclofen dose was chosen on the basis of the results of a series of preliminary experiments demonstrating that it was the highest ineffective dose (in terms of sedation/hypnosis) in DBA mice (this laboratory, unpublished results).

**Fig. 2.** Effect of COR627 and COR628 on the stimulation of \( ^{35}\text{S} \)GTPγS binding via GABAB receptors in rat frontal cortex. COR627 and COR628 were tested alone or in combination with GABA (A), baclofen (at the submaximal concentration of 10 µM) (B), or the competitive antagonist of the GABAB receptor, CGP54626 (B). Horizontal dotted lines indicate baseline values and the degree of stimulation with agonist alone, respectively. Data are from a typical experiment, performed in triplicate and are expressed as mean disintegrations per minute values with S.E.M.

**Fig. 3.** Effect of COR627 and COR628 on morphine-induced (A) and L-glutamate-induced (B) stimulation of \( ^{35}\text{S} \)GTPγS binding in rat striatal and frontal cortex membranes. Horizontal dotted lines indicate baseline values and the degree of stimulation with agonist alone, respectively. Data shown are from a typical experiment performed in triplicate, expressed as mean disintegrations per minute values with S.E.M.
In both experiments and according to the procedure used in previous studies (Carai et al., 2004), after baclofen injection each mouse was placed on its back once every 60 s until it was unable to right itself within 60 s. The time between baclofen injection and the start of the 60-s interval during which the mouse was unable to right itself was measured as onset of LORR. Each mouse was then left undisturbed on its back until it spontaneously regained its righting reflex (determined as having at least three paws under its body). Complete recovery of righting reflex was defined as the animal being able to turn itself upright twice more within 60 s. If this criterion was not fulfilled, the mouse was left undisturbed until it spontaneously regained its righting reflex. The time between loss and recovery of righting reflex was monitored in each mouse and defined as duration of LORR. Observations were conducted by an operator unaware of the drug treatment.

In both experiments, the occurrence of LORR was evaluated by a \( \chi^2 \) test. Data on onset and duration (both expressed in minutes) of LORR were evaluated by separate one-way ANOVAs, followed by the Newman-Keuls test for post hoc comparisons; mice that did not lose their righting reflex were assigned the values of 60-min onset and 0-min duration, respectively.

**Pentobarbital Studies.** Two independent experiments were conducted, one testing COR627 and one testing COR628. These experiments were conducted to assess the specificity of the potentiating effect of COR627 and COR628 on baclofen-induced sedation/hypnosis. COR627 (0, 30, 100, and 300 mg/kg) and COR628 (0, 30, 100, and 300 mg/kg) were administered intraperitoneally 30 min before the injection of a fixed dose of 5-ethyl-5-(pentan-2-yl)-1,3-diazinane-2,4,6-trione (pentobarbital) (20 mg/kg i.p.) to independent groups of \( n = 7 \) to 8 mice each. COR627 and COR628 were suspended in saline with a few drops of Tween 80. Pentobarbital was dissolved in saline. All drugs were injected at a volume of 12.5 ml/kg. The pentobarbital dose was chosen on the basis of the results of a series of preliminary experiments demonstrating that it was minimally effective (in terms of sedation/hypnosis) in DBA mice (this laboratory, unpublished results).

In both experiments, onset and duration of LORR were recorded as described above. Occurrence of LORR was evaluated by a \( \chi^2 \) test. Data on onset and duration (both expressed in minutes) of LORR were evaluated by separate one-way ANOVAs; mice that did not lose their righting reflex were assigned the values of 60-min onset and 0-min duration, respectively.

**Results**

**Identification of COR627 and COR628 by Virtual Screening.** COR627 was identified by means of a virtual screening protocol, according to a procedure recently adopted by some of us for other targets (see Brogi et al., 2009, 2011).

Six structurally diverse GABA<sub>B</sub> PAMs were selected from the literature (Urwyler et al., 2001; Guery et al., 2007; Malherbe et al., 2008; Bauer et al., 2010) to define the essential features required to exert the expected pharmacological activity (the pharmacophore). The best hypothesis consisted of five features (Fig. 1B): three hydrophobic sites (H1–H3), one aromatic group (R), and one hydrogen-bond donor (D and vector).

The pharmacophore model was used to perform a virtual screening of two databases (ASINEX Gold Collection and ChemBridge EXPRESS-Pick Collection) to identify molecules sharing equivalent steric and electronic features located at an appropriate distance from each other. Among them, compound 5571990 from the ChemBridge EXPRESS-Pick Collection was one of the compounds with the best fitness with respect to the common features pharmacophore identified by this query (Fig. 1B). For pharmacological studies, both compound 5571990 (COR627) and its simplified analog COR628 were produced by chemical synthesis in house as reported under Materials and Methods.

**Effects of COR627 and COR628 on Agonist-Induced Stimulation of \( [\text{35S}] \text{GTP} \gamma \text{S} \) Binding.** As shown in Fig. 2A, GABA at 10 and 100 \( \mu M \), the latter being the maximally active concentration, stimulated \( [\text{35S}] \text{GTP} \gamma \text{S} \) binding to rat cortex membranes to approximately 140 and 160\%, respectively, of the basal activity. In the presence of 10 \( \mu M \) GABA, 2.5 and 25 \( \mu M \) COR627 and COR628 potentiated GTP\( \gamma \)S stimulation induced by GABA alone (Fig. 2A). At 10 \( \mu M \) GABA, a slightly, although significantly, enhanced \( [\text{35S}] \text{GTP} \gamma \text{S} \) stimulation to levels higher than those produced by the saturating concentration of 100 \( \mu M \) GABA was observed for COR627 (25 \( \mu M \), but not COR628 (Fig. 2A). Likewise, COR627 and COR628 increased the agonistic effect of a submaximal concentration (10 \( \mu M \)) of baclofen (Fig. 2B). On the other hand, in the absence of GABA or baclofen, neither COR627 nor COR628 produced any stimulation of \( [\text{35S}] \text{GTP} \gamma \text{S} \) binding above baseline levels (Fig. 2, A and B). When tested in the absence of GABA, neither compound stimulated basal \( [\text{35S}] \text{GTP} \gamma \text{S} \) binding up to 100 \( \mu M \) (data not shown). COR627 and COR628 produced no GTP\( \gamma \)S stimulation when the baclofen-induced activation was blocked by CGP54626, a competitive antagonist of the GABA<sub>B</sub> receptor (Fig. 2B).

To verify the selectivity of enhancing GABA<sub>B</sub>-stimulated \( [\text{35S}] \text{GTP} \gamma \text{S} \) binding, COR627 and COR628 were incubated with either morphine (10 \( \mu M \)) or different concentrations of L-glutamate. As shown in Fig. 3, A and B, COR627 and

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<th>Compounds</th>
<th>Conc.</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; GABA ( \mu M )</th>
<th>Maximal Effect Relative to</th>
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<tr>
<td>( 0 ) (control)</td>
<td></td>
<td>4.87 ± 0.70</td>
<td>GABA Control, Basal Activity</td>
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<tr>
<td>COR627</td>
<td>5</td>
<td>2.32 ± 0.06***</td>
<td>100</td>
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<tr>
<td>COR627</td>
<td>10</td>
<td>1.96 ± 0.20***</td>
<td>110 ± 2</td>
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<tr>
<td>COR627</td>
<td>30</td>
<td>1.13 ± 0.21***</td>
<td>120 ± 4***</td>
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<tr>
<td>COR628</td>
<td>5</td>
<td>3.71 ± 0.89</td>
<td>197 ± 4***</td>
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<tr>
<td>COR628</td>
<td>10</td>
<td>1.98 ± 0.46**</td>
<td>105 ± 2</td>
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<tr>
<td>COR628</td>
<td>30</td>
<td>1.26 ± 0.21**</td>
<td>111 ± 3***</td>
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* \( P < 0.05 \), compared with GABA alone (Dunnett’s test).
** \( P < 0.01 \).
*** \( P < 0.001 \).
COR628 failed to potentiate morphine- and L-glutamate-induced [35S]GTPγS binding to striatal or cortical rat membranes, respectively.

**Effects of COR627 and COR628 on Agonist Potency and Efficacy in the [35S]GTPγS Binding Assay.** To characterize the positive allosteric modulatory profile of COR627 and COR628, GABA concentration-response curves were performed in the absence and presence of different fixed concentrations of both compounds. GABA stimulated [35S]GTPγS binding in a concentration-dependent manner with an EC$_{50}$ value of 4.87 ± 0.70 μM and a maximal stimulation of 165 ± 3% of the basal values (Table 1). Increasing the fixed concentrations of COR627 or COR628 induced a leftward shift of the GABA concentration-response curve with only a slight concomitant increase in maximal GABA stimulation (Fig. 4, A and B). As seen in Table 1, in the presence of 30 μM COR627 and COR628, the EC$_{50}$ for GABA decreased by approximately 4-fold, whereas the increase in $E_{\text{max}}$ reached its maximal stimulating effect at the concentration of 10 μM. No further increase in GABA $E_{\text{max}}$ potentiation was observed at 30 μM COR627 and COR628. Increasing the concentrations of COR627 and COR628 in the presence of fixed concentrations of GABA (1 and 10 μM) modulated the agonistic effect of GABA with EC$_{50}$ values in the low micromolar range (COR627: EC$_{50}$ 1.7 ± 0.37 and 0.91 ± 0.21 μM in the presence of 1 and 10 μM GABA, respectively; COR628: EC$_{50}$

![Fig. 4](image-url). Concentration-response curves for GABA in the [35S]GTPγS binding in the absence (○) and presence of COR627 (▲) and COR628 (●) 5 μM; △, 10 μM; ▼, 30 μM). GABA responses were measured in rat cortical membranes. Data shown are from a typical experiment performed in triplicate, expressed as mean disintegrations per minute values with S.E.M. above basal activity. Basal activity (above nonspecific binding) in the representative experiments shown here was 20,000 dpm. The parameters describing the different curves are given in Table 1.

![Fig. 5](image-url). Effects of COR627 (A) and COR628 (B) on GABA-induced [35S]GTPγS binding in membranes from rat cortex. The potentiating effect of COR627 and COR628 was measured at two fixed concentrations of GABA, 1 μM (●) and 10 μM (■); the corresponding control levels, measured in the presence of GABA alone, are indicated by horizontal lines (○, 1 μM GABA; □, 10 μM GABA). The upper line (◇) represents the level of maximal stimulation obtained by a saturating concentration (100 μM) of GABA alone. Data shown are from a typical experiment performed in triplicate, expressed as mean disintegrations per minute values with S.E.M. above basal activity. Basal activity (above nonspecific binding) in the representative experiments shown here was 20,000 dpm.
3.6 ± 0.35 and 0.97 ± 0.24 μM in the presence of 1 and 10 μM GABA, respectively (Fig. 5, A and B).

**Effects of COR627 and COR628 on [3H]CGP54626 Binding.** COR627 and COR628 were further characterized by competition binding using [3H]CGP54626. As expected, CGP54626 caused complete inhibition of the specific binding of [3H]CGP54626 with an IC50 of 3.12 ± 0.05 nM, whereas both COR627 and COR628, up to a concentration of 1 mM, failed to modify [3H]CGP54626 binding (data not shown).

To determine the effects of COR627 and COR628 on the affinity of GABA for GABA\textsubscript{B} receptors, [3H]CGP54626 displacement curves were performed in the presence of a fixed concentration (30 μM) of COR627 and COR628. As shown in Fig. 6, displacement of [3H]CGP54626 from native GABA\textsubscript{B} receptors by GABA was significantly better fitted by a two-site binding model (F test; GraphPad Prism), revealing two populations of binding sites, with high and low affinity, respectively. The affinities of both high- and low-affinity components of agonist binding were increased (F \textsubscript{2,13} = 6.67, P = 0.0127 and F \textsubscript{2,13} = 10.37, P = 0.0029, for high- and low-affinity sites, respectively) by 30 μM COR627 and COR628 (Fig. 6; Table 2), not changing the relative proportions of the two affinity components.

**Effect of COR627 and COR628 on Baclofen-Induced LORR in Mice.** Pretreatment with COR627 resulted in a dose-dependent potentiation of the sedative/hypnotic effect produced by treatment with the subthreshold dose of 35 mg/kg baclofen. Analysis of quantal data indicated that the number of mice that lost their righting reflex was 0 of 6, 1 of 6, 2 of 6, 3 of 6, 5 of 6, and 6 of 6 in mouse groups pretreated with 0, 3, 10, 30, 100, and 300 mg/kg COR627, respectively (χ\textsuperscript{2} = 17.94, df = 5, P < 0.005). Onset of LORR was dose-dependently reduced by pretreatment with COR627 (F \textsubscript{5,30} = 11.72, P < 0.0001) (Fig. 7A), whereas duration was dose-dependently increased by pretreatment with COR627 (F \textsubscript{5,30} = 14.86, P < 0.0001) (Fig. 7B). Pretreatment with COR628 resulted in a dose-dependent potentiation of baclofen-induced sedation/hypnosis. Analysis of quantal data indicated that the number of mice that lost their righting reflex was 2 of 6, 0 of 6, 0 of 6, 1 of 6, 4 of 6, and 6 of 6 in mouse groups pretreated with 0, 3, 10, 30, 100, and 300 mg/kg COR628, respectively (χ\textsuperscript{2} = 25.79, df = 5, P < 0.0001).

Onset of LORR was dose-dependently reduced by pretreatment with COR628 (F \textsubscript{5,30} = 18.88, P < 0.0001) (Fig. 8A), whereas duration was dose-dependently increased by pretreatment with COR628 (F \textsubscript{5,30} = 15.79, P < 0.0001) (Fig. 8B).

**Effect of COR627 and COR628 on Pentobarbital-Induced LORR in Mice.** Pretreatment with COR627 failed to alter the sedative/hypnotic effect produced by treatment with 20 mg/kg pentobarbital. Analysis of quantal data indicated that the number of mice that lost their righting reflex was 2 of 8, 2 of 7, 2 of 7, and 2 of 7 in mouse groups pretreated with 0, 30, 100, and 300 mg/kg COR627, respectively (χ\textsuperscript{2} = 0.04, df = 3, P > 0.05). Neither onset (F \textsubscript{3,25} = 0.18, P > 0.05) nor duration (F \textsubscript{3,25} = 0.53, P > 0.05) of LORR was altered by pretreatment with COR627 (Table 3).

Pretreatment with COR628 failed to alter the sedative/hypnotic effect produced by treatment with 20 mg/kg pentobarbital. Analysis of quantal data indicated that the number of mice that lost their righting reflex was 2 of 8, 1 of 7, 1 of 7, and 1 of 7 in mouse groups pretreated with 0, 30, 100, and 300 mg/kg COR628, respectively (χ\textsuperscript{2} = 0.47, df = 3, P > 0.05). Neither onset (F \textsubscript{3,25} = 0.58, P > 0.05) nor duration (F \textsubscript{3,25} = 0.11, P > 0.05) of LORR was altered by pretreatment with COR628 (Table 4).

### Discussion

In the present study, the authors report the identification of two novel thiophene derivatives, COR627 and COR628, that act as GABA\textsubscript{B} PAMs in rat cortical membranes. Both compounds potentiated GABA- and baclofen-induced signals in an [35S]GTP\textsubscript{S} binding assay, a well validated functional receptor test. Furthermore, the lack of [35S]GTP\textsubscript{S} binding stimulation by both compounds in the absence of an agonist clearly indicates how COR627 and COR628 are GABA\textsubscript{B} PAMs devoid of intrinsic agonistic activity. The modulatory effects of COR627 and COR628 on GTP\textsubscript{S} stimulation were blocked by CGP54626, a competitive antagonist of the

### Table 2

Effects of COR627 and COR628 on the displacement of [3H]CGP54626 by GABA in rat cortical membranes

<table>
<thead>
<tr>
<th></th>
<th>High-Affinity IC\textsubscript{50} (nM)</th>
<th>High-Affinity Site</th>
<th>Low-Affinity IC\textsubscript{50} (μM)</th>
<th>Low-Affinity Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA</td>
<td>27.0 ± 10.0</td>
<td>44.6 ± 3.3</td>
<td>3.4 ± 0.4</td>
<td>55.4 ± 3.4</td>
</tr>
<tr>
<td>GABA + COR627</td>
<td>2.6 ± 1.0*</td>
<td>39.4 ± 4.3</td>
<td>1.8 ± 0.3*</td>
<td>60.6 ± 4.4</td>
</tr>
<tr>
<td>GABA + COR628</td>
<td>4.9 ± 1.7*</td>
<td>45 ± 5.3</td>
<td>1.4 ± 0.2**</td>
<td>54.8 ± 5.4</td>
</tr>
</tbody>
</table>

* P < 0.05, compared with GABA alone (Dunnett’s test).

** P < 0.01.
GABA$_B$ receptor, pointing to the involvement of the agonist site of the GABA$_B$ receptor in such effects.

Of note, neither compounds enhance the stimulation of $[^{35}S]$GTP$_S$ binding to striatal and cortical rat membranes produced by morphine and L-glutamate, suggesting that COR627 and COR628 were selective GABA$_B$ PAMs.

In our hands, concentration-response curves with COR627 and COR628 in the presence of fixed concentrations of GABA (1 and 10 $\mu$M) revealed a potentiating effect of both compounds on GABA-stimulated $[^{35}S]$GTP$_S$ binding to rat cortical membranes with EC$_{50}$ values in the micromolar range. Likewise, when concentration-response curves were measured for GABA at different fixed concentrations of COR627 and COR628, both compounds showed an enhancement of GABA effects at native GABA$_B$ receptors. Compared with the few available in vivo effective GABA$_B$ PAMs (namely CGP7930, GS39783, and rac-BHFF), the potency of COR627 and COR628 was comparable to that of CGP7930 (Urwyler et al., 2001) and GS39783 (Urwyler et al., 2003) but lower than that of rac-BHFF (Malherbe et al., 2008). In addition, the COR627- and COR628-induced increase in agonist $E_{max}$ values was 20 and 10%, respectively, lower than that induced by CGP7930 (Urwyler et al., 2001), rac-BHFF (Malherbe et al., 2008), and GS39783 (Urwyler et al., 2003). Taken together, these data demonstrate that COR627 and COR628 effect mainly the affinity/potency of GABA rather than its efficacy, in contrast to the extensively characterized CGP7930, GS39783, and rac-BHFF (Urwyler et al., 2001, 2003; Malherbe et al., 2008), which modulate both potency and maximal activity of GABA.

Allosteric modulators may affect either 1) agonist potency but not efficacy as reported for muscarinic receptors (Birdsall et al., 1999) or 2) both potency and efficacy (Langmead and Christopoulos, 2006), as described for mGlu1, adenosine A3, and GABA$_B$ (Urwyler, 2011) receptors. This latter mechanism of allosteric modulation is accounted for by the extension of the two-state model of receptor activation (Hall, 2000).

The GABA$_B$ PAM profile of COR627 and COR628 on native GABA$_B$ receptors was further confirmed in radioligand experiments. When the radioligand $[^{3}H]$CGP54626 was displaced by GABA alone or in the presence of 30 $\mu$M COR627 and COR628, all inhibition curves were biphasic, presumably because of the presence of GABA$_B$ receptors coupled to and uncoupled from their G proteins. Moreover, $[^{3}H]$CGP54626 binding site was positively modulated by COR627 and COR628; in agreement with data from CGP7930 and GS39783 (Urwyler et al., 2001, 2003; Asay and Boyd, 2006). COR627 and COR628 enhanced the affinities for GABA of both receptor forms (high- and low-affinity sites) without modifying the relative proportions of the two affinity components. Thus, in contrast with the prototypical GABA$_B$ PAM, GS39783, our compounds did not promote the interactions of the receptor with the G-protein. Moreover, COR627 and COR628 failed to displace $[^{3}H]$CGP54626 binding.

Taken together, the results of the in vitro experiments indicate that COR627 and COR628 display positive allosteric modulatory properties at the GABA$_B$ receptor. The lack of intrinsic activity (GTPyS binding) and interaction with the orthosteric binding site should result in the lack of any unwanted side effects typical of GABA$_B$ receptor agonists.

Therefore, an additional step in this investigation was to evaluate the effect of COR627 and COR628 in an in vivo test. To this end, the potentiating effect of COR627 and COR628 on baclofen-induced sedation/hypnosis in mice was assessed.

**Fig. 7.** Effect of COR627 on the sedative/hypnotic effect of the GABA$_B$ receptor agonist, baclofen, in DBA mice. Onset (A) and duration (B) of LORR, respectively, after administration of COR627 and baclofen. COR627 (0, 3, 10, 30, 100, and 300 mg/kg i.p.) was administered 30 min before administration of baclofen (35 mg/kg i.p.). Each bar is the mean $\pm$ S.E.M. of n = 6 mice. Mice that did not lose the righting reflex were assigned the values of 60-min onset and 0-min duration, respectively. *, $P < 0.01$; **, $P < 0.005$ in comparison with the “0 mg/kg COR627 plus 35 mg/kg baclofen” mouse group (Newman-Keuls test).

**Fig. 8.** Effect of COR628 on the sedative/hypnotic effect of the GABA$_B$ receptor agonist baclofen in DBA mice. Onset (A) and duration (B) of LORR, respectively, after administration of COR628 and baclofen. COR628 (0, 3, 10, 30, 100, and 300 mg/kg i.p.) was administered 30 min before administration of baclofen (35 mg/kg i.p.). Each bar is the mean $\pm$ S.E.M. of n = 6 mice. Mice that did not lose the righting reflex were assigned the values of 60-min onset and 0-min duration, respectively. *, $P < 0.005$ in comparison with the “0 mg/kg COR628 plus 35 mg/kg baclofen” mouse group (Newman-Keuls test).
Of note, potentiation of the sedative/hypnotic effect of baclofen or other \( \text{GABA}_B \) receptor agonists constitutes a reliable test with which to assess the in vivo potential of putative \( \text{GABA}_B \) PAMs (Carai et al., 2004; Malherbe et al., 2008; Koek et al., 2010).

The results of the in vivo experiments demonstrate how pretreatment with per se ineffective doses of COR627 and COR628 synergistically increased the sedative/hypnotic effect of a subthreshold dose of baclofen. In both experiments with baclofen, all three parameters of baclofen-induced sedation/hypnosis [in particular, 1) proportion of mice losing the righting reflex 2) onset of LORR, and 3) duration of LORR] were indeed markedly and dose-dependently altered by pretreatment with COR627 and COR628. Comparison of the data generated by these two experiments indicated no difference in efficacy between COR627 and COR628, because the highest tested dose of both drugs (300 mg/kg) produced LORR in all mice, displaying similar reductions and increases in onset and duration of LORR, respectively. In contrast, COR627 tended to be more potent than COR628, as some degree of potentiation of baclofen-induced sedation/hypnosis was observed at doses of COR627 as low as 3 and 10 mg/kg, whereas the lowest dose of COR628 potentiating baclofen-induced sedation/hypnosis was 30 mg/kg. A possible reason for this difference may reside in different pharmacokinetic properties between the two drugs. Finally, the potentiating effect of COR627 and COR628 was specific for baclofen, because no dose of both COR627 and COR628 altered, even minimally, the sedative/hypnotic effect of pentobarbital. Taken together, these in vivo data are consistent with those previously collected with CGP7930 (Carai et al., 2004; Malherbe et al., 2008; Koek et al., 2010) and rac-BHFF (Malherbe et al., 2008; Koek et al., 2010), thus emphasizing how COR627 and COR628 exhibit a \( \text{GABA}_B \) PAM behavior also in an in vivo assay.

**TABLE 3**

Effect of COR627 on the sedative/hypnotic effect of pentobarbital in DBA mice

<table>
<thead>
<tr>
<th>COR627</th>
<th>No of Rats Losing the Righting Reflex</th>
<th>Onset of LORR (min)</th>
<th>Duration of LORR (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg/kg</td>
<td>2/8</td>
<td>48.5 ± 7.5</td>
<td>4.1 ± 3.5</td>
</tr>
<tr>
<td>30 mg/kg</td>
<td>2/7</td>
<td>46.7 ± 8.6</td>
<td>3.9 ± 2.5</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>2/7</td>
<td>47.4 ± 8.1</td>
<td>7.3 ± 4.8</td>
</tr>
<tr>
<td>300 mg/kg</td>
<td>2/7</td>
<td>45.8 ± 8.4</td>
<td>13.3 ± 7.8</td>
</tr>
</tbody>
</table>

**TABLE 4**

Effect of COR628 on the sedative/hypnotic effect of pentobarbital in DBA mice

<table>
<thead>
<tr>
<th>COR628</th>
<th>No of Rats Losing the Righting Reflex</th>
<th>Onset of LORR (min)</th>
<th>Duration of LORR (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg/kg</td>
<td>2/8</td>
<td>49.9 ± 6.7</td>
<td>6.3 ± 4.7</td>
</tr>
<tr>
<td>30 mg/kg</td>
<td>1/7</td>
<td>57.4 ± 2.6</td>
<td>3.6 ± 2.6</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>1/7</td>
<td>53.6 ± 6.4</td>
<td>3.9 ± 3.9</td>
</tr>
<tr>
<td>300 mg/kg</td>
<td>1/7</td>
<td>57.8 ± 2.0</td>
<td>8.0 ± 7.4</td>
</tr>
</tbody>
</table>

In conclusion, the findings of the present study demonstrate that the two novel compounds, COR627 and COR628, act as positive allosteric modulators of \( \text{GABA}_B \) receptors, thereby constituting additional tools for use in investigating \( \text{GABA}_B \) receptor function and physiopathology.

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**Authorship Contributions**

**Participated in research design:** Castelli, Colombo, Tafi, Gessa, and Corelli.

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**Performed data analysis:** Castelli, Casu, Casti, Lobina, and Carai.

**Wrote or contributed to the writing of the manuscript:** Castelli, Colombo, Gessa, and Corelli.

**References**

Asay MJ and Boyd SK (2006) Characterization of the binding of \( [\text{H}^3] \text{CGP54628} \) to \( \text{GABA}_B \) receptors in the male bullfrog \( (\text{Rana catesbeiana}) \). Brain Res 1094:76–85.


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