Coronaridine congeners potentiate GABA_A receptors and induce sedative activity in mice in a benzodiazepine-insensitive manner

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Running title: Sedative activity and PAM activity on GABAARs of coronaridine congeners

Abbreviations: LORR, loss of righting reflex; O-maze, elevated zero maze; GABA; y-

aminobutyric acid; Gly, glycine; GABAAR, GABA type A receptor; GlyR, glycine receptor; PAM,

positive allosteric modulator; (±)-18-MC, (±)-18-methoxycoronaridine; (+)-catharanthine, (+)-

3,4-didehydrocoronaridine; RT, room temperature; BZD, benzodiazepine; EC₅₀, agonist

concentration that produces 50% of maximal response; potentiating EC₅₀, ligand concentration

that produces half-maximal potentiation response; n_H, Hill coefficient; ED₅₀, ligand dose that

produces half-maximal response; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal

bovine serum.

Keywords: Coronaridine congeners; Sedative activity; Positive allosteric modulators; GABAA

Receptors; Glycine Receptors.

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Abstract

To determine whether (+)-catharanthine induces sedative- or anxiolytic/anxiogenic-like activity in male mice, proper animal paradigms were used. The results showed that (+)-catharanthine induces sedative-like activity in the 63-72 mg/Kg dose range in a flumazenil-insensitive manner, but neither this effect nor anxiolytic/anxiogenic-like activity was observed at lower doses. To determine the underlying molecular mechanism of the sedative-like activity, electrophysiological and radioligand binding experiments were performed with (+)-catharanthine and (±)-18methoxycoronaridine [(±)-18-MC] on GABA_A (GABA_ARs) and glycine receptors (GlyRs). Coronaridine congeners both activated and potentiated a variety of human (h) GABAARs, except hp1. (+)-Catharanthine-induced potentiation followed this receptor selectivity (EC₅₀'s in μM): $h\alpha 1\beta 2$ (4.6 ± 0.8) > $h\alpha 2\beta 2\gamma 2$ (12.6 ± 3.8) ~ $h\alpha 1\beta 2\gamma 2$ (14.4 ± 4.6) indicating that both $\alpha 1$ and $\alpha 2$ are equally important, whereas $\gamma 2$ is not necessary. (+)-Catharanthine was >2-fold more potent and efficient than (\pm) -18-MC at h α 1 β 2 γ 2. (+)-Catharanthine also potentiated, whereas (\pm) -18-MC inhibited, hal GlyRs with very low potency. Additional [3H]-flunitrazepam competition binding experiments using rat cerebellum membranes clearly demonstrated that these ligands do not bind to the benzodiazepine site. This is supported by the observed activity at $h\alpha 1\beta 2$ (lacking the BDZ site) and similar effects between α 1- and α 2-containing GABA_ARs. Our study shows, for the first time, that (+)-catharanthine induced sedative-like effects in mice, and coronaridine congeners potentiated human $\alpha 1\beta 2\gamma 2$, $\alpha 1\beta 2$, and $h\alpha 2\beta 2\gamma 2$, but not $\rho 1$, GABA_ARs, both in a benzodiazepine-insensitive fashion, whereas only (+)-catharanthine slightly potentiated GlyRs.

Introduction

Coronaridine congeners, including (-)-18-methoxycoronaridine (18-MC), ibogaine, and noribogaine decrease self-administration of many different abused drugs in animal models, making them significant lead compounds for the development of anti-addictive therapies (Maisonneuve and Glick, 2003). Ibogaine (Brown and Alper, 2018) and its active metabolite, noribogaine (Glue et al., 2016), alleviate drug craving and decrease relapse during opioid detoxification in humans. Based on animal results showing that 18-MC produces anti-addictive activity without the side effects found in ibogaine (Glick et al., 1999), this synthetic compound is currently under clinical trial by Savant HWP.

Although coronardine congeners may act on several potential targets, making them relatively unspecific compounds (Glick et al., 1999), (±)-18-MC has higher selectivity for α3β4-containing nicotinic acetylcholine receptors (α3β4* AChRs) compared to other AChR subtypes (Arias et al., 2015; Arias et al., 2017) which is the basis for its safer profile (Glick et al., 1999). The current notion is that the anti-addictive activity of coronaridine congeners is mediated by α3β4* AChRs expressed in the habenula-interpeduncular pathway, which modulates the brain reward system located in the corticolimbic area (Glick et al., 2011). However, there are other potential targets that have not been studied yet, including GABA_A (GABA_ARs) and glycine receptors (GlyRs). GlyRs and GABA_ARs, two major inhibitory receptor members from the Cys-loop ligand-gated ion channel superfamily where AChRs also belong, are expressed in brain areas important for learning and memory mechanisms (Xu and Gong, 2010), as well as brain reward and addiction processes (Riegel and Kalivas, 2010). Structurally different compounds behave as positive allosteric modulators (PAMs; i.e., potentiate receptor activity in the presence of an agonist) of GABA_ARs, including neurosteroids, benzodiazepines (BZDs), several general anesthetics,

barbiturates, and a variety of natural compounds (Choi et al., 1981; Miller et al., 2017; Shen et al., 2012; Puthenkalam et al., 2016). BZDs are used to treat clinical conditions such as anxiety, convulsions, muscle spasms, and insomnia, whereas dihydromyricetin decreases alcohol intoxication and withdrawal effects (Shen et al., 2012). On the other hand, GlyRs are potential targets for drugs with analgesic, anti-inflammatory, and antidepressant (e.g., ginkgolic acids) activity (Maleeva et al., 2015).

In this regard, an important objective of this work is to characterize the pharmacological activity of (+)-catharanthine and (\pm)-18-MC (see Fig. 1 for its molecular structures) on heteromeric $\alpha1\beta2\gamma2$, $\alpha1\beta2$, and $\alpha1\beta2\gamma2$ as well as homomeric hp1 GABAARs, and homomeric ha1 GlyR. We selected the $\alpha1\beta2\gamma2$ GABAAR because it is the most abundant receptor subtype in the brain (Sigel and Steinmann, 2012; Miller et al., 2017). To gain a better insight on the active site for coronaridine congeners, we also used the $\alpha1\beta2$ GABAAR to determine the role of the $\gamma2$ subunit (i.e., BZD site) (Sieghart, 2015), and the p1 GABAAR to assess the involvement of allosteric binding sites for general anesthetics, barbiturates, and neurosteroids, which are lacking in p-containing GABAARs (Choi et al., 1981; Shen et al., 2012; Sigel and Steinmann, 2012; Sieghart, 2015). Since previous studies suggested that BZD modulators induce sedative- or anxiolytic-like activity depending on their different selectivity for $\alpha1$ - and $\alpha2$ -containing GABAARs (Low et al., 2000; McKernan et al., 2000; Tan et al., 2011), we also compared ligand activity between $\alpha2\beta2\gamma2$ and $\alpha1\beta2\gamma2$ GABAARs.

Animal results suggested that coronaridine congeners have antidepressant, anxiolytic, antiobesity (Maisonneuve and Glick, 2003), and anti-neuropathic pain (submitted manuscript) activities. However, there is no study demonstrating whether coronaridine congeners have sedative-like activity and its underlying molecular mechanisms. In this regard, an important objective of this work is to determine the sedative- and anxiolytic/anxiogenic-like activity of (+)-catharanthine in male mice by using animal paradigms such as loss of righting reflex (LORR) test (sedative activity) (Katayama et al., 2007) and elevated zero maze (O-maze) test (anxiolytic/anxiogenic activity) (Shepherd et al., 1994). The open field test was also used to determine the effect of (+)-catharanthine on locomotor activity (Weisstaub et al., 2006). To determine whether the (+)-catharanthine-induced sedative-like activity is mediated by direct interaction with the BZD site at GABAARs, two approaches were used: flumazenil-induced inhibition of the observed sedative-like activity, and [³H]-flunitrazepam competition binding using rat cerebellum membranes containing abundant amounts of BZD sites.

The main results of this work demonstrated that (+)-catharanthine produces sedative-like activity in male mice in a BZD-insensitive fashion, and that (+)-catharanthine and (\pm)-18-MC potentiate human $\alpha 1\beta 2\gamma 2$, $\alpha 1\beta 2$, and $\alpha 1\beta 2\gamma 2$, but not $\alpha 1\beta 2\gamma 2$, but not $\alpha 1\beta 2\gamma 2$, but not $\alpha 1\beta 2\gamma 2$, and $\alpha 1\beta 2\gamma 2$, but not $\alpha 1\beta 2\gamma 2$, and $\alpha 1\beta 2\gamma 2$, but not $\alpha 1\beta 2\gamma 2$, but no

2. Material and Methods

2.1. Material

[³H]-Flunitrazepam (sp. act. 83 Ci/mmol) was purchased from Perkin Elmer New England Nuclear (Waltham, Massachusetts, USA). Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4, benzodiazepine-2-one) was obtained from Nycomed (Opfikon, Switzerland). Glycine (Gly), γ-aminobutyric acid (GABA), penicillin (5000 U/mL)-streptomycin (5 mg/mL), fetal bovine serum (FBS), poly-L-lysine hydrobromide, Dulbecco's Modified Eagle Medium (DMEM), and cOmpleteTM protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO, USA). (±)-18-Methoxycoronaridine hydrochloride [(±)-18-MC] was purchased from Obiter Research, LLC (Champaign, IL, USA). (+)-Catharanthine hydrochloride was a gift from

Dr. Kuehne (University of Vermont, VT, USA), and its quality previously tested (Arias et al., 2017). (+)-Catharanthine (base) was obtained from Cayman Chemical (Ann Arbor, MI, USA). Flumazenil was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). pHiSpeed® plasmid purification kit was obtained from QIAGEN Inc. (Valencia, CA, USA). TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) was purchased from Bioexpress (Layton, UT, USA). PolyJetTM DNA *In Vitro* transfection reagent was obtained from SignaGen Laboratories (Rockville, MD, USA). *Trans*-IT 293 transfection reagent was purchased from Mirus, Bio LLC (Madison, WI, USA). Salts, solvents, and reagents were purchased from commercial suppliers and used as received.

2.2. Behavioral Experiments

2.2.1. Animals

Adult male Swiss albino CD1 mice (20-22 g) purchased from Janvier Labs (Le Genest Saint Isle, France) were used in the study. The animals were housed in Makrolon cages (L: 37 cm, W: 21 cm, H: 14 cm), with free access to standard semisynthetic laboratory diet (SERLAB, Montataire, France). All animals were kept in a ventilated room, at a temperature of 22 ± 1 °C, under a 12-h light/12-h dark cycle (light between 7:00 a.m. and 7:00 p.m.).

All behavioral experiments were carried out between 9:00 a.m. and 6:00 p.m. in testing rooms adjacent to the animal rooms and were conducted by authorized investigators. Mice were tested only once and sacrificed immediately. Animal manipulations were approved by Reginal Ethical Committee for Animal Experimentation and performed according to the European Communities Council Directive (86/609/EEC + 2010/63/UE).

2.2.2. Elevated Zero Maze (O-Maze) Test

The O-maze test was used to determine the anxiogenic/anxiolytic-like activity of (+)-catharanthine (base) in male mice. This test is based on the rodent's aversion to elevated and open spaces involving a conflicting response between exploration and aversion (Shepherd et al., 1994). In this regard, the percentage of open-arm entries and the time or frequency spent on open arms provide appropriate measures of animal anxiety, while the number of closed-arm entries constitutes a better measurement of locomotor activity.

The apparatus consisted of an infrared circular black Plexiglas platform (outer diameter 45 cm, width 6 cm), placed 60 cm above the ground level in a dimly illuminated room, divided equally into four quadrants with two open opposite, and two equal closed (were surrounded by 27 cm wall from the surface of the maze) quadrants. 15 min before the experiments, the animals (n = 10/condition) were isolated in small individual cages (L 25 cm, W 9 cm, H 8 cm) in a testing room, at an ambient temperature of 22 ± 1°C. Thirty minutes after the i.p. injection of (+)-catharanthine (20 or 36 mg/Kg) [dissolved in vehicle: DMSO (1%), Tween 80 (5%), NaCl (0.9%)] or vehicle, each animal was placed at the end of the open section with the head facing a closed quadrant. The used doses were those not inducing sedative effect by using the open field test. The number of entries, the time spent and the distance travelled in open and closed quadrants were recorded for 5 min using an automated image analysis system (Ethovision XT v13.0, Noldus Information Technologies, Wageningen, The Netherlands). The animal was considered be in the open or closed quadrant when the gravity center of its image (registered to the camera set around 2 m above the maze) was detected in each of these regions.

2.2.3. Loss of righting reflex (LORR) test

The LORR test was used to measure the sedative-like effects of (+)-catharanthine (base) on male mice. In this regard, the time required for the animal to right itself through 180 $^{\circ}$ was

measured and expressed as immobility time (Katayama et al., 2007). Animals (n = 10/condition) were injected (i.p.) with vehicle or different doses of (+)-catharanthine (20, 36, 50, 63, or 72 mg/Kg). Thirty minutes after the injection, when mice become motionless, they were placed individually on their back in a position of dorsal recumbence in a plexiglass V-shaped trough until they were able to right themselves within 10 min. The experimenter then observed the mice until they turned over onto all of four paws-defined as righting themselves. Immobile time, corresponding to the sedative effect, was defined as the time from being placed in the supine position until the animal regained their righting reflexes.

To determine whether the sedative-like effect elicited by (+)-catharanthine is mediated by GABA_ARs through a "BZD-like" mechanism, 3 mg/Kg flumazenil [dissolved in vehicle: 2% DMSO, 0.9% NaCl] was administered 30 min prior (+)-catharanthine (72 mg/Kg), and the LORR test subsequently performed. The dose of 3 mg/Kg flumazenil, a BZD antagonist, was chosen based on previous studies (Ishola et al., 2012).

2.2.4. Open Field Test

The open field test (Weisstaub et al., 2006) was used to determine the effect of different doses of (+)-catharanthine (base) (20, 36, 50, 63, or 72 mg/Kg) on locomotor activity. This test was assessed automatically in a computerized actimeter (Versamax, AccuScan Instruments, Inc., OH, USA) which monitored horizontal displacements and vertical movements, including rearing, leaning and jumping. The animals (n = 10/condition) were placed individually in 20 x 20 x 30 cm compartments, 10 min after i.p. injection of vehicle or (+)-catharanthine, in a dimly illuminated and quiet room. The locomotor activity was measured for a period of 60 min to determine the total time of the locomotor effect, whereas measurements were performed every 10 min to determine the onset time for the locomotor effect.

To determine whether the effect of (+)-catharanthine on locomotor activity is mediated by GABA_ARs through a "BZD-like" mechanism, 3 mg/Kg flumazenil was administered 30 min prior (+)-catharanthine (63 mg/Kg), and the open field test subsequently performed.

2.3. Patch-clamp recording of HEK293 cells expressing human GABAARs or GlyRs

Human (h) $\alpha 1\beta 2\gamma 2$ and $h\alpha 2\beta 2\gamma 2$ GABA_ARs stably expressed in HEK293 cells were generously provided by John Drewe (Cytovia Inc, San Diego, CA). The pharmacological properties of these receptors have been extensively characterized in our previous publications (Huang et al., 2001; Huang et al., 2004; Huang et al., 2017). On the other hand, $h\alpha 1\beta 2$ and $h\rho 1$ GABA_ARs, and $h\alpha 1$ GlyRs, were transiently transfected in HEK293 cells using PolyJetTM DNA *In Vitro* and *Trans*-IT 293 transfection reagents (Snell and Gonzales, 2015; Chen et al., 2017). Briefly, $h\rho 1$ (2 μg with 2 μg GFP), $h\alpha 1\beta 2$ (1 μg $\alpha 1$, 2 μg $\beta 2$) GABA_ARs, or $h\alpha 1$ GlyRs (1 μg) subunit cDNAs were added to cells growing on glass coverslips. Cells that showed expression of the green fluorescent protein were subjected to patch-clamp electrophysiology, as described previously (Snell and Gonzales, 2015). Transfected cells were used for electrophysiological analysis 24-48 h after the transfection.

Whole-cell patch clamp recordings were performed at room temperature (RT) (22-25 °C) at a holding potential of -60 (h α 1/2 β 2 γ 2 and h α 1 β 2 GABA_ARs, and h α 1 GlyRs) or -70 mV (h ρ 1). Patch pipettes of borosilicate glass (M1B150F, World Precision Instruments, Inc., Sarasota, FL, USA) were pulled using a P-87 Flaming/Brown or a Sutter P-97 horizontal puller (Sutter Instrument Co., Novato, CA) to a tip resistance of 6-9 M Ω . The pipette solution for the h α 1/2 β 2 γ 2 GABA_ARs and h α 1 GlyRs contained (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 4 Mg-ATP; pH 7.2. A coverslip containing transfected cells was placed in a small chamber (~1.5 mL) on the stage of an upright fixed stage microscope (Nikon OPtiphot-2UD) and superfused

continuously (5-8 mL/min) with the following external solution containing (in mM): 125 NaCl, 5.5 KCl, 0.8 MgCl₂, 3.0 CaCl₂, 10 HEPES, 10 glucose, pH 7.3. The external solution for the hp1 GABAAR contained (in mM): 137 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 HEPES; pH 7.4, whereas the internal recording solution contained: 120 CsCl, 20 tetraehtylammonium chloride, 1 CaCl₂, 2 MgCl₂, 11 EGTA, and 10 HEPES; pH 7.2 (Snell and Gonzales, 2015). In this case, a Perfusion Fast-Step 77B (Warner Instrument LLC., Hamden, CT, USA) equipped with an array of square capillary tubes was used.

GABA or Gly was prepared in extracellular solution and applied (10-s) to cells containing the GABA_ARs or GlyRs *via* gravity flow using a Y-shaped tube positioned near the target cell. With this system, the 10-90% rise time of the junction potential at the open tip was 60-120 ms (Huang and Dillon, 1999). In the case of the hp1 GABA_AR, the GABA control and coronaridine congener containing test solutions were applied for 5 s. A washout period of 90 s followed each recording to ensure a return to baseline current readings was achieved before proceeding to the next recording. Receptor currents were obtained using a patch clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA, USA) equipped with a CV201A headstage, whereas a pClamp10.0 (Molecular Devices, Sunnyvale, CA, USA) on an inverted fluorescence microscope equipped with a FITC fluorescence cube was also used to record hp1 GABA_AR currents.

The GABA_AR and GlyR currents were low-pass filtered at 5 kHz, monitored on an oscilloscope and a chart recorder (Gould TA240), sampled at 30 KHz using a Digidata 1440A and stored on a computer (pClamp 10.6, Molecular Devices) for subsequent analysis. To determine the PAM activity of (+)-catharanthine and (±)-18-MC (both HCl salts), receptors were typically activated with non-saturating agonist concentrations corresponding approximately to its

EC₃₀ values. More precisely, 3 μ M (h α 1 β 2), 5 μ M (h ρ 1), 10 μ M (h α 1 β 2 γ 2), or 20 μ M GABA (h α 2 β 2 γ 2), or 50 μ M Gly (h α 1) (Huang et al., 2001; Huang et al., 2004; Huang et al., 2017). These concentrations were chosen because they produce minimal receptor desensitization, which may confound interpretation of results. To monitor the possibility that access resistance changed over time or during different experimental conditions, the current response to a -5 mV voltage pulse was measured at the initiation of each recording and stored on our digital oscilloscope. This stored trace was continually referenced throughout the recording. If a change in access resistance was observed throughout the recording period, the patch was aborted and the data were not included in the analysis.

The concentration-response relationship for the activity of coronaridine congeners on GABA_ARs and GlyRs was analyzed using the following logistic equation (Potasiewicz et al., 2015):

$$I_{PAM}/I = 1/[1 + (potentiating EC50/[Ligand])^{n_H}]$$
 (1)

where I is the current amplitude determined in the absence of any coronaridine congener (control assigned as 100%), I_{PAM} is the potentiation induced by each coronaridine congener [Ligand], potentiating EC_{50} is the concentration of each coronaridine congener producing half-maximal potentiation, and n_H is the Hill coefficient.

2.4. [³H]-Flunitrazepam Competition Binding Assay

Rat cerebellum was used to prepare membranes containing abundant amounts of BZD-containing GABA_ARs (<u>Pöltl</u> et al., 2003) for the subsequent radioligand binding competition experiments. Tissue from rat cerebellum was homogenized with an Ultra Turrax for 30 s in ice-cold homogenization buffer (10 mM Hepes, 1 mM EDTA, 300 mM sucrose, and protease inhibitor cocktail, pH 7.5) and centrifuged at 45,000 x g for 30 min at 4 °C. The pellet was

resuspended in wash buffer (10 mM Hepes, 1 mM EDTA, and protease inhibitor cocktail pH 7.5), incubated on ice for 30 min and pelleted as described above. The pellet was stored at -80 °C overnight, and the next day, washed five times with 50 mM Tris-citrate buffer (pH = 7.1), and subsequently centrifuged as described above. Membrane pellets were stored at -80 °C until final use.

Frozen membranes were thawed, suspended (500 μ L) and incubated for 90 min at 4 °C in 50 mM Tris/citrate buffer (pH = 7.1), containing 150 mM NaCl and 2 nM [³H]-flunitrazepam, in the absence or presence of 5 μ M diazepam. To determine the effect of coronaridine congeners on [³H]-flunitrazepam binding to cerebellum membranes, 50 μ M of (+)-catharanthine or (±)-18-MC [dissolved in DMSO (0.5% final concentration)] was added to the membrane suspension. Membranes were filtered through Whatman GF/B filters and the filters rinsed twice with 4 mL of ice-cold 50 mM Tris/citrate buffer. Filters were transferred to scintillation vials and subjected to scintillation counting after the addition of 3 ml Rotiszint Eco plus liquid scintillation cocktail. Specific [³H]-flunitrazepam binding was calculated by subtracting nonspecific binding (5 μ M diazepam) from total binding (no diazepam).

2.5. Statistical Analysis

The functional data (mean ± SEM) were analyzed by using Origin 6.0 (Microcal Software, Northampton, MA, USA). The unpaired Student's t-test was used to compare the functional activity of both congeners. The animal study data (mean ± SEM) were analyzed by using the Prism software (GraphPad 6.0 Software Inc., La Jolla, CA, USA). Multiple comparisons were performed using ANOVA, where the mean value from each drug-treated animal group was compared to that for the vehicle-treated animal group, and this initial analysis was followed by Dunnett's post-hoc tests. The Tukey's post-hoc test was particularly used in the interaction

studies between (+)-catharanthine and flumazenil. The multiplicity adjusted probability value was reported for each comparison, where p < 0.05 was considered significantly different.

3. Results

3.1. (+)-Catharanthine induces sedative- but not anxiolytic/anxiogenic-like effects in mice

To determine the anxiolytic/anxiogenic-like effects mediated by coronaridine congeners, the activity of (+)-catharanthine was investigated by using the O-maze test (Fig. 2). The results showed no significant difference between animals treated with vehicle or (+)-catharanthine at doses of 20 or 36 mg/Kg. More precisely, ANOVA analyses followed by Dunnett's test indicated that (+)-catharanthine did not produce any statistically significant effect at either 20 mg/Kg [F (2, 54) = 0.061; p = 0.9405] or 36 mg/Kg [F (2,54) = 0.043; p = 0.9579].

The sedative-like effect of (+)-catharanthine at different doses was subsequently investigated by using the LORR test (Fig. 3A). Our findings indicated that (+)-catharanthine, compared to vehicle-treated animals, significantly produced a loss of righting reflex at 63 mg/Kg [F (5, 54) = 43.96; p < 0.0001], but not at lower doses, producing the longest possible immobility time (10 min) at 72 mg/Kg [F (5, 54) = 43.96; p < 0.0001]. Interestingly, ANOVA analyses followed by Tukey's test indicated that the co-administration of 3 mg/Kg flumazenil did not antagonize the sedative-like effect elicited by (+)-catharanthine (p = 0.8489) (Fig. 3B).

Our open field test results showed that (+)-catharanthine decreases locomotor activity at high (63 and 72 mg/Kg) (Fig. 4A), but not low (20 and 36 mg/Kg), doses. The latter is in agreement with previous experiments showing that 40 mg/Kg (\pm)-18-MC did not change rat's locomotor activity (Glick et al., 1999). To study the observed effect in more detail, several locomotor parameters were measured every 10 min. In general, (+)-catharanthine decreased horizontal locomotor activity compared to that for vehicle-treated animals (p < 0.01). The effect

started during the first minutes, indicating a quick onset, and lasted for 39 min of the test, wearing off after that time. ANOVA analyses followed by Dunnett's test indicated that 63 mg/Kg (+)-catharanthine decreased several locomotor parameters (72 mg/Kg gave similar statistical results), including horizontal activity [0-10 and 10-20 min: F (5, 360) = 26.22, p < 0.0001; 20-30 min: F (5, 360) = 75.46, p < 0.0001] (Fig. 4A), total distance [0-10 min: F (5, 360) = 36.81, p < 0.0001; 10-20 and 20-30 min: F (5, 360) = 45.89, p < 0.0001] (Fig. 4B), rest time [0-10 min: F (5, 360) = 42.64, p < 0.001; 10-20 and 20-30 min: F (5, 360) = 42.64, p < 0.0001] (Fig. 4C), vertical activity [0-10 min: F (5, 360) = 8.565, p < 0.0001; 10-20 and 20-30 min: F(5, 360) = 37.86, p < 0.0001 (Fig. 4D), vertical time [0-10, 10-20, and 20-30 min: F(5, 360) = 37.86, p < 0.0001 (Fig. 4D), vertical time [0-10, 10-20, and 20-30 min: F(5, 360) = 37.86, p < 0.0001 (Fig. 4D), vertical time [0-10, 10-20, and 20-30 min: F(5, 360) = 37.86, p < 0.0001 (Fig. 4D), vertical time [0-10, 10-20, and 20-30 min: F(5, 360) = 37.86, p < 0.0001 (Fig. 4D), vertical time [0-10, 10-20, and 20-30 min: F(5, 360) = 37.86, p < 0.0001 (Fig. 4D), vertical time [0-10, 10-20, and 20-30 min: F(5, 360) = 37.86, p < 0.0001 (Fig. 4D), vertical time [0-10, 10-20, and 20-30 min: F(5, 360) = 37.86, p < 0.0001 (Fig. 4D), vertical time [0-10, 10-20, and 20-30 min: F(5, 360) = 37.86, p < 0.0001 (Fig. 4D), vertical time [0-10, 10-20, and 20-30 min: F(5, 360) = 37.86, p < 0.0001 (Fig. 4D), vertical time [0-10, 10-20, and 20-30 min: F(5, 360) = 37.86, p < 0.0001 (Fig. 4D), vertical time [0-10, 10-20, and 20-30 min: F(5, 360) = 37.86, p < 0.0001 (Fig. 4D), vertical time [0-10, 10-20, and 20-30 min: F(5, 360) = 37.86, p < 0.0001 (Fig. 4D), vertical time [0-10, 10-20, and 20-30 min: F(5, 360) = 37.86, p < 0.0001 (Fig. 4D), vertical time [0-10, 10-20, and 20-30 min: F(5, 360) = 37.86, p < 0.0001 (Fig. 4D), vertical time [0-10, 10-20, and 20-30 min: F(5, 360) = 37.86, p < 0.0001360) = 46.48, p < 0.001] (Fig. 4E), and movement time [0-10 and 10-20 min: F (5, 360) = 41.54, p < 0.001; 20-30 min: p = 0.8239) (Fig. 4F). Interestingly, the co-administration of 3 mg/Kg flumazenil did not antagonize the decreased locomotor activity effects elicited by (+)catharanthine within the first 30-min timespan, and flumazenil alone did not produce any effect by itself (Fig. 5). More precisely, ANOVA analyses followed by Tukey's test gave the following values at 0-10 min (p = 0.3532), 10-20 and 20-30 min (p = 0.9999), respectively, for the horizontal activity (Fig. 5A). The same applies to other motor activity parameters, including total distance (Fig. 5B), rest time (Fig. 5C), vertical activity (Fig. 5D), vertical time (Fig. 5E), and movement time (Fig. 5F).

3.2. Agonistic and potentiating activities of coronaridine congeners at different human $GABA_AR$ subtypes

To determine the molecular mechanism underlying the sedative-like effect elicited by (+)-catharanthine (see Fig. 3), the pharmacological activity of coronaridine congeners was compared among $h\alpha 1\beta 2\gamma 2$, $h\alpha 1\beta 2$, $h\alpha 2\beta 2\gamma 2$, and $h\rho 1$ GABA_AR subtypes.

To determine whether coronaridine congeners have direct activity on different GABAARs, the agonistic activity of 300 μ M (+)-catharanthine, tested on $h\alpha1\beta2\gamma2$, $h\alpha1\beta2$, and $h\alpha2\beta2\gamma2$ GABAAR subtypes, and of 300 μ M (\pm)-18-MC, tested on $h\alpha1\beta2\gamma2$, was compared to that elicited by saturating concentrations of GABA (i.e., 1 mM for $h\alpha1/2\beta2\gamma2$; 500 μ M for $h\alpha1\beta2$) (Figs. 6A,B). Furthermore, (+)-catharanthine's direction action on $h\alpha1\beta2\gamma2$ was detectable at 30 μ M and evident at concentrations above 100 μ M (data not shown). The results indicated that (+)-catharanthine activated several GABAARs with different efficacy, whereas (\pm)-18-MC elicited a neglectable effect (1.3 \pm 0.4 %) on $h\alpha1\beta2\gamma2$ GABAARs even at the highest used concentration (i.e., 300 μ M). The agonistic efficacy of (+)-catharanthine at $h\alpha1\beta2$ (42.0 \pm 7.9 %) was much higher than that at either $h\alpha1\beta2\gamma2$ (14.5 \pm 2.2 %) or $h\alpha2\beta2\gamma2$ (8.9 \pm 2.7 %) (p < 0.05; unpaired Student's t-test) (Fig. 6B; Table 1), supporting a deleterious effect mediated by $\gamma2$.

To assess whether coronaridine congeners behave as positive allosteric modulators (PAMs) of GABA_ARs, (\pm)-18-MC (3-300 μ M) and (+)-catharanthine (1-300 μ M) were respectively co-applied with GABA at concentrations corresponding to the EC₃₀ value for each receptor subtype. Both congeners potentiated different GABA_ARs in a concentration-dependent manner (Fig. 7A). At higher concentrations, however, (+)-catharanthine started to inhibit GABA-activated currents as indicated by the reduced amplitude and rebound current, where the threshold concentration depended on the receptor subtype (e.g., >30 μ M for h α 1 β 2; >100 μ M for h α 1 β 2 γ 2 and h α 2 β 2 γ 2) (Fig. 7A). The concentration-response curves showed that (+)-catharanthine's PAM activity (potentiating EC₅₀ values) depended on the receptor subtype (Fig. 7B; Table 1): h α 1 β 2 (4.6 \pm 0.8 μ M) > h α 2 β 2 γ 2 (12.6 \pm 3.8 μ M) ~ h α 1 β 2 γ 2 (14.4 \pm 4.6 μ M) (unpaired Student's t-test; p < 0.05), and the calculated efficacies (Δ E_{max}) followed a similar trend: h α 1 β 2 (209 \pm 18 %) ~ h α 1 β 2 γ 2 (178 \pm 31 %) (p > 0.05) > h α 2 β 2 γ 2 (118 \pm 26 %) (p <

0.05). In particular, (+)-catharanthine showed higher efficacy and potency at h α 1 β 2 γ 2 GABA_ARs, compared to (±)-18-MC (83 ± 12 % and 29.6 ± 5.4 μ M, respectively; p < 0.05) (Table 1). Interestingly, neither (+)-catharanthine (Fig. 7C) nor (±)-18-MC (Fig. 7D) were able to enhance hp1 GABA_ARs, whereas (+)-catharanthine slightly inhibited (~30 % at 300 μ M) this receptor subtype.

The calculated n_H values indicated that although (+)-catharanthine potentiated $h\alpha 1\beta 2$ and $h\alpha 2\beta 2\gamma 2$ GABA_ARs, and (±)-18-MC potentiated $h\alpha 1\beta 2\gamma 2$ GABA_ARs, with values higher than unity, suggesting a cooperative mechanism, in agreement with the fundamental mechanism of PAMs, (+)-catharanthine potentiated $h\alpha 1\beta 2\gamma 2$ GABA_ARs with a value near unity, supporting a non-cooperative mechanism (Table 1).

3.3. (+)-Catharanthine is a positive allosteric modulator of homomeric hal GlyRs

To examine whether coronaridine congeners affect hal GlyR activity, different concentrations of (+)-catharanthine (Fig. 8A) and (\pm)-18-MC (Fig. 8B) were compared to that elicited by a saturating concentration of Gly (i.e., 1 mM). The results indicated that neither (+)-catharanthine nor (\pm)-18-MC directly activated hal GlyRs, even at concentrations as high as 300 μ M. However, when (+)-catharanthine was co-applied with a non-saturating concentration of Gly (50 μ M), it potentiated the receptor in a concentration-dependent manner. The calculated efficacy and potency for the PAM activity of (+)-catharanthine was 83 \pm 32% (at 300 μ M) and 106 \pm 27 μ M, respectively (Table 1). The n_H value (near unity) for (+)-catharanthine suggests a non-cooperative mechanism (Table 1). On the contrary, (\pm)-18-MC slightly inhibited hal GlyRs at the highest testing concentrations, giving an estimated IC50 value of 407 \pm 134 μ M (Table 1).

3.3. Coronaridine congeners do not bind to the benzodiazepine site

To demonstrate whether coronaridine congeners bind to the BZD site at GABA_ARs, [3 H]-flunitrazepam competition binding assays were performed using rat cerebellum membranes. Rat cerebellum expresses high levels of $\alpha 1\beta 1\gamma 2$ GABA_ARs, the most abundant subtype in the brain containing the BZD site (Pöltl et al., 2003).

The results from three experiments, each performed in duplicate, clearly demonstrated that neither (+)-catharanthine (95.3 \pm 3.9 %) nor (\pm)-18-MC (87.7 \pm 2.9 %) inhibited [3 H]-flunitrazepam binding (Fig. 9).

4. Discussion

The objective of the present study is to determine whether (+)-catharanthine exhibits anxiolytic/anxiogenic- and/or sedative-like activity in male mice, and the pharmacological mechanism underlying the observed sedative-like activity by characterizing the pharmacological activity of coronaridine congeners at different GABAAR and GlyR subtypes.

To first determine the anxiolytic/anxiogenic-like activity of (+)-catharanthine, the Omaze test was used in male mice. No significant differences were observed between animals treated with either (+)-catharanthine or vehicle, supporting neither anxiolytic- nor anxiogenic-like activity in the 20-36 mg/Kg dose range. These results support a previous statement by McCallum and Glick (2009), suggesting that since (±)-18-MC has no rewarding nor aversive effects, its activity on cocaine conditioned place preference is mediated neither by its anxiolytic-nor anxiogenic-like effects. The open field test results also indicated that (+)-catharanthine does not induce any locomotor/anxiolytic activity at low doses, but decreased mice's locomotor activity at higher doses. This latter effect had a quick onset and lasted for ~30 min, in contrast with the anti-addictive activity of (±)-18-MC (40 mg/Kg) which lasted for ~100 min (Glick et al., 1999) without decreasing locomotor activity in rats (McCallum and Glick, 2009), suggesting that

the *in vivo* metabolism/excretion of (+)-catharanthine is faster than that for (\pm)-18-MC. Although the decreased mice's locomotor activity elicited by (+)-catharanthine could be mediated by ganglionic $\alpha 3\beta 4^*$ AChR inhibition, the comparatively lower potency (~39-fold) of (+)-catharanthine in inhibiting muscle-type AChRs (Arias et al., 2011) ruled out the possibility of a sedative-like effect mediated by muscle relaxation.

To determine whether (+)-catharanthine produces sedative-like activity on mice, the LORR test, an animal paradigm better suited for this purpose, was used as well. Our results indicated a prolonged duration of loss of righting reflex elicited by (+)-catharanthine, supporting a sedative-like effect at doses of 56 and 72 mg/Kg. The observed activity contrasts with the lack of sleeping/sedative-like effects in rats by (\pm)-18-MC at doses as high as 100 mg/Kg (Glick et al., 1999). These different effects correlate very well with their PAM activities, where (+)-catharanthine's potency and efficacy at h α 1 β 2 γ 2 GABAARs are double than that for (\pm)-18-MC. This is the first time showing coronaridine congener-induced sedative-like activity in rodents. The fact that (+)-catharanthine starts producing mild sedative-like effects at 36 mg/Kg, becoming more prominent at \geq 50 mg/Kg, precluded us to determine whether this congener has anxiolytic-like activity at doses higher than 36 mg/Kg.

To determine the molecular mechanism underlying the observed sedative-like effect, subsequent electrophysiological experiments were performed using a variety of GABAAR and GlyR subtypes. The overall results indicated that coronaridine congeners produced agonistic and PAM activities on a variety of GABAARs, following this receptor selectivity: $h\alpha1\beta2 > h\alpha1\beta2\gamma2$ ~ $h\alpha2\beta2\gamma2$, indicating that the observed PAM activity is $\gamma2$ -independent and does not discriminate between $\alpha1$ - and $\alpha2$ -containing GABAARs. On the other hand, (+)-catharanthine potentiated, whereas (±)-18-MC slightly inhibited, $h\alpha1$ GlyRs. Our study is in agreement with

previous results showing that ibogaine, another coronaridine congener, did not interact with the GABA_AR ion channel, ruling out potential blocking mechanisms (Deecher et al., 1992).

The lack of PAM activity at homomeric hp1 GABAARs shown in our experiments supports the concept that coronaridine congeners might be producing its pharmacological effects by interacting with any of the allosteric binding sites (i.e., BZD, neurosteroid, and/or general anesthetic/barbiturate sites) found at GABAARs (Choi et al., 1981; Shen et al., 2012; Sigel and Steinmann, 2012; Sieghart, 2015). Although our experimental results cannot define the exact mechanism of action and the functional site(s) involved in the sedative-like activity elicited by (+)-catharanthine showed in the present study, some suggestions can be provided. First, the possibility that GlyRs may mediate the sedative-like effect elicited by (+)-catharanthine can be ruled out based on the observed very low potency. Second, based on the relatively very low efficacy of (+)-catharanthine to directly activate GABAARs, a mechanism involving (+)catharanthine-induced direct stimulation can also be ruled out. Instead, our results add new pieces of evidence supporting the view that coronaridine congeners potentiate GABAARs by interacting with an allosteric site different to that for BZDs. This statement is based on the stronger PAM and direct effects observed on h α 1 β 2 GABA_ARs, receptor subtype lacking the γ 2 subunit, which is essential for BZD activity (Sieghart, 2015). And, also supported by the observed (+)-catharanthine-induced PAM activity at $\alpha 1$ GlyRs, in contrast to true BZD derivatives that did not enhance agonist-induced GlyR activity (Choi et al., 1981). Previous studies have demonstrated that BZD modulators induce sedative- or anxiolytic-like activity depending on their different selectivity for α1- and α2-containing GABA_ARs (Low et al., 2000; McKernan et al., 2000; Tan et al., 2011), whereas (+)-catharanthine induced both PAM and direct actions on both GABA_AR subtypes.

The strongest evidence against a BZD-like mechanism is provided by two experiments using BZD antagonists. The results with flumazenil indicated that this BZD antagonist failed to inhibit the (+)-catharanthine-induced sedative-like activity in mice. These results are similar to that for U-90042, which displayed both binding affinity for $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ GABA_ARs and sedative-like activity (Tang et al., 1995). Interestingly, flumazenil, which binds to the BZD site at $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ GABA_AR subtypes (Tang et al., 1995), failed to inhibit U-90042-induced locomotor affects. In this regard, our results can be interpreted in two different ways: catharanthine's sedative-like effect is mediated by GABAARs but not through a BZD-like mechanism, or this effect is independent of its PAM activity on GABAARs. To finally discriminate between these two possible mechanisms, [3H]-flunitrazepam competition binding experiments were performed using cerebellum membranes containing high amounts of BZDsensitive $\alpha 1\beta 2\gamma 2$ GABA_ARs. Our results clearly demonstrated that neither (+)-catharanthine nor (±)-18-MC decreased [³H]-flunitrazepam binding to GABA_AR-containing membranes, indicating that these two compounds do not bind to the BZD site at $\alpha 1\beta 2\gamma 2$ GABA_ARs. In addition to the possibility that (+)-catharanthine may induce sedation via unidentified mechanisms such as other GABAAR subtypes and allosteric sites distinct to the BZD site, it may also be implied that (+)catharanthine induces sedation via GABAARs expressed in neural circuits different from that where BZDs produce its particular effects (Tan et al., 2011).

Our study shows, for the first time, that coronaridine congeners produce sedative-like effects in male mice in a BZD-insensitive manner. Our results also suggest that this effect is mediated by a mechanism involving BZD-insensitive GABAAR potentiation, ruling out GlyR potentiation. Nevertheless, other allosteric mechanisms might be considered, including those for compounds such as neurosteroids and/or general anesthetics/barbiturates. Notably, each one of

these compounds has agonistic and PAM activities on GABA_ARs (Miller et al., 2017) and PAM effects on GlyRs (Maksay et al., 2001), as well as sedative-like activity in animals and human beings (Campagna et al., 2003). Since these pharmacological and behavioral characteristics are relatively alike to those observed for (+)-catharanthine, it is plausible that this congener shares the functional site for either of these allosteric modulators at GABA_ARs. To learn more about plausible binding sites for coronaridine congeners at GABA_ARs, we compared the binding sites for these compounds at α 3 β 4 AChRs (Arias et al., 2015) with known allosteric sites at GABA_ARs (Choi et al., 1981; Shen et al., 2012; Sigel and Steinmann, 2012; Sieghart, 2015), and found a site located in the α 3 β 4 AChR transmembrane domain, between α 3-M2/3 and β 4-M1, that partially overlaps the propofol/etomidate site at GABA_ARs situated in a cavity formed at the β 3(+)/ α 1(-) interface. We are planning further investigations to elucidate the exact molecular mechanism(s) underlying coronaridine congeners' sedative-like effects.

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Table 1. Pharmacologic activity of coronaridine congeners at different GABA_ARs and $h\alpha 1$ GlyRs, determined by whole-cell patch clamp recordings.

Receptor Subtype	Coronaridine Congener (concentration range)	Agonistic ^a (at 300 µM) or Antagonistic Activity	PAM Activity	Potentiating EC ₅₀ or IC ₅₀ b (µM)	ΔE_{max}^{c}	n _H ^d
hα1β2 GABA _A R	(+)-Catharanthine (1-300 μM)	42.0 ± 7.9 ª	Yes	4.6 ± 0.8	209 ± 18	1.8 ± 0.7
hα2β2γ2 GABA _A R	(+)-Catharanthine (1-300 μM)	8.9 ± 2.7 ^a	Yes	12.6 ± 3.8	118 ± 26	2.2 ± 0.8
hα1β2γ2 GABA _A R	(+)-Catharanthine (1-300 μM)	14.5 ± 2.2 ^a	Yes	14.4 ± 4.6	178 ± 31	1.3 ± 0.4
hα1β2γ2 GABA _A R	(±)-18-MC (3-300 μM)	1.3 ± 0.4 ^a	Yes	29.6 ± 5.4	83 ± 12	3.6 ± 4.3
hp1 GABA _A R	(+)-Catharanthine (30-300 μM)	Slight inhibition	No	I	-	_
hp1 GABA _A R	(±)-18-MC (30-300 μM)	No activity	No		ı	_
hα1 GlyR	(+)-Catharanthine (10-300 μM)	_	Yes	106 ± 27	83 ± 32	1.2 ± 0.4
hα1 GlyR	(±)-18-MC (10-300 μM)	Slight inhibition	No	407 ± 134 ^b	_	2.1 ± 2.0 b

^a Agonistic activity for each congener was tested at 300 μM, and normalized to the maximal current amplitude elicited by a saturating concentration of GABA (assigned as 100%).

^b Estimated IC₅₀ and its respective n_H value

 $^{^{}c}$ ΔE_{max} , potentiating efficacy for each congener determined from the respective fitting curve. Maximal relative current amplitude [% over the control (set at 100%) in the absence of any congener].

^d Hill coefficients corresponding to the PAM activity.

Figure legend

Figure 1. Molecular structures of (-)-18-methoxycoronaridine [(-)-18-MC] and (+)-catharanthine [(+)-3,4-didehydrocoronaridine].

Figure 2. (+)-Catharanthine produced neither anxiolytic- nor anxiogenic-like activity in male mice determined by elevated zero maze tests. Animals (n = 10/condition) were injected (i.p.) with vehicle (i.e., control) (\square) or (+)-catharanthine at doses of 20 mg/Kg (\square) or 36 mg/Kg (\square), and then placed at the end of the open section, with the head facing a closed quadrant. The time spent (A), the distance traveled (B) and the frequency (C) with which the animal enters into each arm were recorded during 5-min using an automated image analysis system. ANOVA analyses followed by Dunnett's test indicated no significant (ns) differences between vehicle- and (+)-catharanthine-treated animals at doses of 20 mg/Kg (p = 0.9405) and 36 mg/Kg (p = 0.9579), respectively.

Figure 3. (A) (+)-Catharanthine induced sedative-like activity in male mice. Animals (n = 10/condition) were injected with vehicle or (+)-catharanthine at the following doses: 20, 36, 50, 63, and 72 mg/Kg, and the immobility time (i.e., sedative effect) subsequently determined by loss of righting reflex (LORR) tests. ANOVA analyses followed by Dunnett's test indicated that 63 and 72 mg/Kg (+)-catharanthine, but not lower doses, show sedative-like activity compared to vehicle-treated animals (****p < 0.0001). (B) Flumazenil failed to antagonize the sedative-like activity induced by (+)-catharanthine on male mice. Animals (n = 10/condition) were injected with vehicle+vehicle (i.e., control), flumazenil (3 mg/Kg)+vehicle, vehicle+(+)-catharanthine (72 mg/Kg), or flumazenil (3 mg/Kg)+(+)-catharanthine (72 mg/Kg), and the immobility time (i.e., sedative effect) subsequently determined by LORR tests. ANOVA analyses followed by Tukey's tests indicated that flumazenil failed to antagonize the sedative-like effect induced by (+)-catharanthine (ns; p = 0.8489).

Figure 4. (+)-Catharanthine decreased locomotor activity in male mice. Animals (n = 10/condition) were injected (i.p.) with vehicle (i.e., control), or (+)-catharanthine at the following doses: 20, 36, 50, 63, or 72 mg/Kg, and subsequently tracked during 60 min in an open field. (A) Mice treated with (+)-catharanthine at doses of 63 and 72 mg/Kg, but not lower doses, decreased horizontal activity compared to vehicle-treated mice (o). The onset of (+)-catharanthine's activity is very quick (in the first minutes of the test), wearing off after 39 min. In addition, several locomotor parameters were determined every 10 min. ANOVA analyses followed by Dunnett's test indicated that 63 mg/Kg (+)-catharanthine decreased several locomotor parameters (72 mg/Kg gave similar statistical results), including (A) horizontal activity [0-10 and 10-20 min: F(5, 360) = 26.22, p < 0.0001; 20-30 min: F(5, 360) = 75.46, p < 0.0001], (B) total distance [0-10.000]10 min: F (5, 360) = 36.81, p < 0.0001; 10-20 and 20-30 min: F (5, 360) = 45.89, p < 0.0001], (C) rest time $[0-10 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ and } 20-30 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ and } 20-30 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ and } 20-30 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ and } 20-30 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ and } 20-30 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ and } 20-30 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ and } 20-30 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ and } 20-30 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ and } 20-30 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ and } 20-30 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ and } 20-30 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ and } 20-30 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ min: } F(5, 360) = 42.64, p < 0.001; 10-20 \text{ m$ p < 0.0001, (D) vertical activity [0-10 min: F (5, 360) = 8.565, p < 0.0001; 10-20 and 20-30 min: F(5, 360) = 37.86, p < 0.0001, (E) vertical time [0-10, 10-20, and 20-30 min: F(5, 360) =46.48, p < 0.001], and (F) movement time [0-10 and 10-20 min: F(5, 360) = 41.54, p < 0.001;20-30 min: p = 0.8239). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.001.

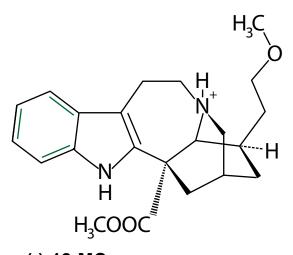
Figure 5. Flumazenil failed to antagonize the decreased motor activity elicited by (+)-catharanthine. Animals (n = 10/condition) were injected with vehicle+vehicle (i.e., control) (, flumazenil (3 mg/Kg)+vehicle (, vehicle+(+)-catharanthine (63 mg/Kg) (, or flumazenil (3 mg/Kg)+(+)-catharanthine (63 mg/Kg), and subsequently tracked during 60 min in an open field. Horizontal activity (A), total distance (B), rest time (C), vertical activity (D), vertical time (E), and movement time (F) parameters were evaluated every 10 min for a total period of 60 min. ANOVA analyses followed by Tukey's test showed no significant (ns) difference between (+)-catharanthine-treated and (+)-catharanthine+flumazenil-treated animal groups at 0-10 min (p = 0.3532), and 10-20, 20-30, 30-40, 40-50, and 50-60 min (p = 0.9999) timespans.

Figure 6. Agonistic activity of coronaridine congeners at GABA_ARs by using whole-cell patch-clamp recordings. (A) GABA_AR currents elicited by direct action of 300 μM (+)-catharanthine [(+)-Cath] (hα1β2γ2, hα1β2, and hα2β2γ2) or 300 μM (±)-18-MC (hα1β2γ2) were compared to the maximal response elicited by saturating concentrations of GABA (i.e., 1 mM for hα1/2β2γ2 and 500 μM for hα1β2). The results indicated that (+)-catharanthine (n = 5), but not (±)-18-MC (n = 8), activated hα1β2γ2 GABA_ARs in the absence of GABA. (B) The agonistic activity of (+)-catharanthine was more evident at hα1β2 GABA_ARs (n = 5) compared to that at either hα1β2γ2 (n = 8) or hα2β2γ2 (n = 5) GABA_ARs (*, unpaired Student's test; p < 0.05). The agonistic activity of each coronaridine congener (mean ± SEM), was normalized to GABA maximal activity (%), and summarized in Table 1.

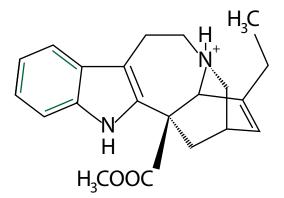
Figure 7. Potentiating activity of coronaridine congeners at GABAARs by using whole-cell patch-clamp recordings. (A) Different ligand concentrations were co-applied for 10 s with nonsaturated concentrations of GABA corresponding to approximately the EC₃₀ value at each GABA_AR subtype. (+)-Catharanthine and (±)-18-MC potentiated a variety of GABA_AR subtypes in a concentration-dependent manner. Moreover, (+)-catharanthine started to inhibit these receptors at higher concentrations indicated by a rebound current, where the threshold concentration depended on the receptor subtype (e.g., >30 μ M for h α 1 β 2; >100 μ M for h α 1 β 2 γ 2 and $h\alpha 2\beta 2\gamma 2$). (B) Concentration-response relationship for (+)-catharanthine at $h\alpha 1\beta 2\gamma 2$ (∇), $h\alpha 1\beta 2$ (\triangle), and $h\alpha 2\beta 2\gamma 2$ (\bullet), and for (\pm)-18-MC at $h\alpha 1\beta 2\gamma 2$ (\bullet) GABA_ARs. The PAM activity of (+)-catharanthine was more evident at $h\alpha 1\beta 2$ GABA_ARs (n = 5) compared to that at $h\alpha 1\beta 2\gamma 2$ (n = 6) or $h\alpha 2\beta 2\gamma 2$ (n = 7) GABA_ARs (unpaired Student's test; p < 0.05). When (+)catharanthine (C) or (±)-18-MC (D) was co-applied for 5 s with a non-saturated concentration of GABA (5 µM) on hp1 GABA_ARs, (±)-18-MC did not show any potentiating activity, while 300 μM (+)-catharanthine was able to slightly inhibit this receptor (n = 3). All current amplitudes (mean \pm SEM) are normalized to the response of GABA in the absence of coronaridine congener (assigned as 100%). The potentiating EC50, n_H , and ΔE_{max} values for each ligand at each receptor subtype were summarized in Table 1.

Figure 8. Pharmacological activity of coronaridine congeners at recombinant hα1 GlyRs. The agonistic activity of (+)-catharanthine (A) and (±)-18-MC (B) was examined at hα1 GlyR by whole-cell patch-clamp recordings. The results indicated that neither (+)-catharanthine nor (±)-18-MC directly activate hα1 GlyRs. The PAM activity of (+)-catharanthine (A) and (±)-18-MC (B) was examined by co-applying a non-saturated concentration of Gly (50 μM) for 10 s. The results indicated that (+)-catharanthine potentiated, while (±)-18-MC inhibited hα1 GlyR currents in a concentration-dependent manner (n = 5-6). Bottom, concentration-activity relationship for the potentiating activity of (+)-catharanthine (A) and for the inhibitory activity of (±)-18-MC (B), respectively. All current amplitudes are normalized to the response of Gly in the absence of any coronaridine congener (assigned as 100%). Each data point represents Mean ± SEM. The EC₅₀, IC₅₀, n_H, and Δ E_{max} values were summarized in Table 1.

Figure 9. Effect of 50 μ M (+)-catharanthine or (±)-18-MC on [³H]-flunitrazepam binding to rat cerebellum membranes containing abundant amounts of BDZ sites. Membranes were incubated for 90 min at 4 °C with (500 μ L) 50 mM Tris/citrate buffer (pH = 7.1), containing 150 mM NaCl and 2 nM [³H]-flunitrazepam in the absence (total binding) and presence of 5 μ M diazepam (non-specific binding). The results (mean ± SEM) from three independent experiments, each performed in duplicate, show that coronaridine congeners do not bind to the benzodiazepine site.



(-)-18-MC



(+)-Catharantine

