Mechanism of action involved in the anxiolytic-like effects of Hibalactone isolated from *Hydrocotyle umbellata* L.

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ABSTRACT

Background and aim: Hibalactone (HB) is a lignan related to the anxiolytic-like effects of *Hydrocotyle umbellata* L. However, there is a need to understand better the mechanism of action of this lignan to support the ethnopharmacological uses of the species. This work aimed to evaluate by *in vivo* and *in silico* analysis the mechanism of action of HB involved in its anxiolytic-like effects.
Experimental procedure: The effects of HB in mice were evaluated on light-dark box (LDB) and elevated plus maze (EPM) tests. The participation of 5-HT$_{1A}$ receptor and the benzodiazepine site of GABA$_A$ receptor was evaluated to investigate the possible mechanism of action. *In silico* tools were used to better elucidate the anxiolytic-like effects of HB.
Results: Oral treatment with HB at a dose of 33 mg/kg showed an anxiolytic-like effect in the LDB and EPM tests. Besides that, the treatment altered the ethological parameters, frequency of head dips, and stretched-attend postures (SAP), important to better describe the anxiolytic profile of HB. Pretreatment with flumazenil (2 mg/kg) reverted the anxiolytic-like effect of HB on LDB and EPM tests. On the other hand, pretreatment with NAN-190 (0.5 mg/kg) not reverted the activity observed. *In silico* predictions revealed the potential of HB to increase GABAergic neurotransmission. Pharmacophore modelling and docking simulations showed that HB might interact with the α1β2γ2 GABA$_A$ receptor.
Conclusion: Together, the results presented herein suggest that activation of the benzodiazepine site of the GABA$_A$ receptor contributes to the anxiolytic-like effect of HB.

1. Introduction

Anxiety disorders are among the most common mental disorders associated with significant disability and impact on the quality of life.[1] The regulation of anxiety in the central nervous system (CNS) is involved with various neurotransmitter systems, mainly the GABAergic system.[2] Anxiolytic drugs, mostly belonging to the benzodiazepines group, are within the most commonly prescribed drugs. However, despite its effectiveness in treating anxiety symptoms, its clinical uses are limited by its potential of abuse, dependence, and withdrawal symptoms.[3,4] Therefore, there is an increasing demand for effective drugs with lesser undesirable effects.[5]

Herbal medicines play a crucial role in human health care. Historically, plants have been a viable source of bioactive compounds with great potential for therapeutic applications in CNS disorders.[6,7] In this context, research efforts have been conducted

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to validate the efficacy of plants commonly used for their anxiolytic properties.[8] Among them is *Hydrocotyle umbellata L.*, Araliaceae, a species native from the American continent with application in Ayurvedic medicine for anxiolytic and memory stimulant effects.[9] Investigations of the biological activities of the species demonstrated the antinociceptive, anti-inflammatory and anxiolytic-like effects of its ethanolic extract and fractions. Moreover, phytochemical screening indicated that these effects might be attributed, at least in part, to the presence of hibalactone.[10]

Hibalactone (HB) (1) is a lignan of the class of dibenzylbutyrolactones found mainly in species of Araliaceae, Cupressaceae, and Rutaceae families.[11] Because hibalactone's therapeutic potential, a study of its extraction optimization and development of an analytical method for its quantification was also proposed in order to contribute to standardization products from *H. umbellata*.[12] However, there is a need to understand the anxiolytic activity of this lignan to support the ethnopharmacological uses of the species. Therefore, this work aimed to evaluate the mechanism of action of hibalactone involved in its anxiolytic-like effects by *in vivo* and *in silico* approaches.

2. Material and methods

2.1. Drugs and chemicals

The reagents used were: Buspirone (Ansitec® - LIBBS Pharmaceutical LTDA, Embu-Guaçu, SP, Brazil); Diazepam (Cristália, Itapira, SP, Brazil); dimethylsulfoxide (10% DMSO, Sigma-Aldrich St. Louis, MO, USA); Flumazenil (União Química, Embu-Guaçu, SP, Brazil); Hibalactone (HB - isolated from *H. umbellata*); NAN-190 (1-(2-methoxyphenyl)-4-[4-(2-(2-phthalimido)butyl]piperazine hydrobromide - Sigma-Aldrich, St. Louis, MO, EUA); Polysorbate 80 (Tween 80® - Sigma-Aldrich, St. Louis, MO, USA); Saline solution (0.9%, NaCl - Belga). The HB was solubilized in 10% DMSO, and then distilled water was added to the desired concentration. The buspirone and diazepam were prepared in distilled water. The NAN-190 was solubilized in 2% Tween 80® and dissolved in 0.9% saline. A 10% DMSO solution in distilled water was used as the vehicle. All compounds were administered at a volume of 10 mL/kg. Doses of the drugs were chosen according to the literature data.[10,13,14]

2.2. Isolation of hibalactone

Samples of *H. umbellata* subterraneous parts were collected in Hidrolândia, (Goiás state of Brazil) located at 16° 40′ 33″ S and 49° 14′ 39′ W at an altitude of 768 m above sea level. The authenticity of the plant material was verified by Dr. José Realino de Paula and a voucher specimen was deposited in the Herbarium of the Universidade Federal de Goiás (UFG-22394). The material was washed with water, desiccated at 40 °C, and ground in a Willye mill. The powdered was stored sheltered from light and moisture. Hibalactone was isolated from the dichloromethane fraction (2 g) of the crude ethanolic extract according to Oliveira et al.,[10] affording 68 mg of pure substance. ^{1}H NMR, ^{13}C NMR, HMBC and HSQC spectral data were used to identify the isolated compound.

2.3. Animals

Behavioral assessments were conducted with male Swiss mice (30–35 g), with nine animals per experimental group. The animals were maintained with free access to water and food, under a 12:12 h controlled light/dark photoperiod cycle (lights on at 7:00 a.m.) and room temperature adjusted to 22 ± 2 °C. All the protocols were approved by the Ethics Commission of the Universidade Federal de Goiás (protocol number 11/2014).

2.4. Evaluation of anxiolytic-like effects of hibalactone

2.4.1. Light-dark box (LDB) test

LDB test is an animal model used in pharmacology to assay unconditioned anxiety response in rodents and was performed as described by Crawley & Goodwin[15] The apparatus consists of two compartments with an opening that allows the animals to transition between both areas. One hour after oral treatments with vehicle (10% DMSO), hibalactone (33 mg/kg), diazepam (1 mg/kg) or buspirone (10 mg/kg), the mice were placed individually at the center of the light area and kept exploring freely for 5 min. The entire session was recorded, and the percentage of time spent in the light area was calculated. Anxiolytic drugs increase this percentage, whereas anxiogenic drugs promote more time in dark area.[16]

2.4.2. Elevated plus maze (EPM) test

EPM test was performed as described by Lister.[17] This test is a widely used behavioral assay for anxiety based on the natural behavior of rodents' possibility the anxiolytic- and anxiogenic-like effects of pharmacological agents can be investigated.[18] The apparatus consists of an elevated maze with four arms (two open and two enclosed) connected by a common central platform. One hour after oral treatments with vehicle (10% DMSO), hibalactone (33 mg/kg), diazepam (1 mg/kg) or buspirone (10 mg/kg), the mice were placed individually at the central platform facing the closed arms. The entire experiment was recorded during 5 min for analysis posterior of the displayed behavioral parameters: total number of entries in the arms of EPM, percentage of open arms entries, percentage of time spent in open arms, percentage of time spent in central platform, frequency of head dips and stretched-attend postures (SAP).

2.4.3. Mechanism of action involved on anxiolytic-like effects

To investigate mechanism of action underlying of anxiolytic-like effects of hibalactone, the participation of $5-HT_{1A}$ receptor and benzodiazepine site of the $GABA_A$ receptor was evaluated. In the experimental session, the animals were pretreated by intraperitoneal administration with 0.9% saline, flumazenil (2 mg/kg, a benzodiazepine site antagonist) or NAN-190 (0.5 mg/kg, a $5-HT_{1A}$ receptor antagonist). After 30 min, the animals were treated orally with vehicle (10% DMSO), hibalactone (33 mg/kg), diazepam (1 mg/kg) or buspirone (10 mg/kg). One hour after the treatment, mice were submitted individually to LDB and EPM tests.

2.5. Statistical analysis

All results were expressed as mean $\pm$ standard error of the mean (SEM), and the statistical difference was considered when $p < 0.05$. The differences between two groups were detected by Student's T-test and among three or more groups by the one-way ANOVA followed by the Newman-Keuls post hoc test.

2.6. In silico studies

2.6.1. Prediction of bioactivity

Bioactivity prediction with the 2D structure of hibalactone was performed using PASS (Prediction of Activity Spectra for Substances)[19] and DIGEP-Pred (Drug-Induced Gene Expression Profiles Prediction)[20] tools. For both methods, Pa and Pi estimate the probability of the compound to be active or inactive, respectively, for each type of activity from the activity database. The activities presenting Pa > Pi were selected for the analysis. Target screening was performed with the SwissTargetPrediction tool, which uses a combination of 2D and 3D similarity with known bioactive compounds.[21]

2.6.2. Pharmacophore modelling

Based on predictions, a search in BindingDB[22] and PubChem[23] databases was conducted to find $\alpha1\beta2\gamma2$ GABA$_A$ ligands. The keyword "GABA$_A$ receptor" was used in the search of bioassay records. The benzimidazole derivatives were selected for the construction of the pharmacophoric model due to their structural similarity with hibalactone. Thus, the pharmacophore aimed to verify if hibalactone shares the stereoelectronic features of benzimidazole derivatives. The ligands were clustered according to their binding affinities and those presenting the lowest IC$_{50}$ values were selected to represent the dataset of ligands (Table A.I).[24–26]

Pharmacophoric models were generated using the PharmaGist webserver.[27] The candidate pharmacophores are detected by multiple flexible alignment of the input ligands. Concerning the parameters, a minimum of 3 features including hydrogen-bond acceptor, hydrogen bond donor, hydrophobic, and ring aromatic features were selected for generating the pharmacophore models. The scoring weight assigned for the features was maintained as default. HB was inputted into the pharmacophore of ligands to obtain the fitting pharmacophore model. The top-scored pharmacophore model was selected.

2.6.3. Docking simulations

Docking simulations of hibalactone and diazepam with Human $\alpha1\beta2\gamma2$ GABA$_A$ receptor (PDB: 6D6U)[28] were performed using the DockThor server.[29] The calculations were carried out with setting of a grid center of $124 \times 170 \times 156$ Å and with a grid size of 20 Å at the benzodiazepine site. The most energetically favorable conformations were selected for the analysis. The advanced options were maintained as default. The docking protocol was validated by redocking the ligand flumazenil in complex with the GABA$_A$. Validation was considered succeeded if the 10 top-ranked redocked orientations showed heavy atoms RMSD values ≤ 2.0 compared with crystallographic orientation.[30]

3. Results

3.1. Anxiolytic-like effect of hibalactone on LDB test

Oral treatment with hibalactone (33 mg/kg) increased the percentage of time spent in the light area of LDB by 22% when compared with vehicle (43.56 ± 1.43) (Fig. 1). The positive controls, diazepam (1 mg/kg) and buspirone (10 mg/kg), also increased this parameter by 32% and 40%, respectively as shown in Fig. 1.

3.2. Anxiolytic-like effect of hibalactone on EPM test

Oral treatments with hibalactone (33 mg/kg), diazepam (1 mg/kg) and buspirone (10 mg/kg) increased the percentage of open arms entries by 28%, 46% and 40%, respectively, when compared with vehicle (39.38 ± 2.34) (Fig. 2a). Neither treatment altered the total number of entries in the EPM arms (Fig. 2b). The percentage of time spent in open arms (Fig. 2c) increased with hibalactone (33 mg/kg), diazepam (1 mg/kg) and buspirone (10 mg/kg) treatments by 46%, 51% and 65%, respectively, when compared with vehicle (27.84 ± 3.41). On the other hand, percentage of time spent in central platform (Fig. 2d) decreased with hibalactone (33 mg/kg), diazepam (1 mg/kg) and buspirone (10 mg/kg) treatments by 35%, 39% and 33%, respectively, when compared with vehicle (29.61 ± 2.23). The ethological parameters have also been changed, demonstrated by increased of head dips frequency with hibalactone (33 mg/kg), diazepam (1 mg/kg) and buspirone (10 mg/kg) treatments by 64%, 173% and 178%, respectively, when compared with vehicle (14 ± 1.01) (Fig. 2e) and decreased of SAP frequency with hibalactone (33 mg/kg), diazepam (1 mg/kg) and buspirone
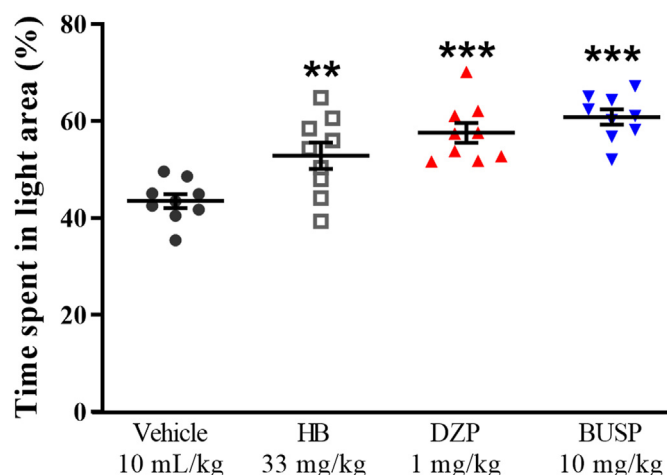

Fig. 1. Anxiolytic-like effects of hibalactone on light-dark box test. Results are expressed as mean $\pm$ SEM (n = 9). Diazepam and buspirone are used as positive controls. **$p \leq 0.01$ and ***$p \leq 0.001$ when compared with vehicle group (10% DMSO, 10 mL/kg) according to one-way ANOVA followed by Newnman-Keuls post hoc test. HB, hibalactone; DZP, diazepam; BUSP, buspirone.

(10 mg/kg) treatments by 47%, 90% and 56%, respectively, when compared with vehicle (24.11 ± 2.62) (Fig. 2f).

3.3. Mechanism of action involved on anxiolytic-like effects

The anxiolytic-like effects of hibalactone (33 mg/kg) demonstrated on LDB and EPM tests were reverted by pretreatment with flumazenil (2 mg/kg), but not with NAN-190 (0.5 mg/kg) (Figs. 3–5). In the LDB test, the percentage of time spent in the light area decreased by 26% in the group flumazenil + hibalactone when compared with the group saline + hibalactone (52.92 ± 2.78) (Fig. 3a). In the EPM, the percentage of open arms entries (Fig. 4a) and percentage of time spent in the open arms (Fig. 5a) decreased by 20% and 17%, respectively, in the group flumazenil + hibalactone when compared with the group saline + hibalactone (50.43 ± 1.64; 40.54 ± 2.05). The pretreatments with flumazenil and NAN-190, *per se*, did not change the observed parameters (Figs. 3–5). However, the doses used with both antagonists, flumazenil and NAN-190, reverted the anxiolytic effects of diazepam (1 mg/kg) (Figs. 3b, 4b and 5b) and buspirone (10 mg/kg) (Figs. 3c, 4c and 5c), respectively.

3.4. In silico studies

The prediction of HB biological activity with the Pass tool indicated that it might be a neurotransmitter uptake inhibitor, specifically for GABA, and GABA aminotransferase inhibitor. Prediction of HB-induced gene expression profile with DIGEP-Pred indicated that it could up-regulate the gene GABARAPL1 (GABA receptor-associated protein like 1). Target prediction with SwissTargetPrediction revealed that HB might interact with $\alpha1\beta2\gamma2$ GABA$_A$ receptor (Table 1).

Pharmacophore modelling of $\alpha1\beta2\gamma2$ GABA$_A$ benzimidazole derivatives ligands is presented in Fig. A.1 (a) and it showed that the main shared features are two aromatic rings and one hydrogen bond acceptor. The presence of aromatic rings and hydrogen bond acceptors in lactone moiety of HB structure favored its fit in the pharmacophoric features of ligands, as showed in Fig. A.1 (b). A greater part of HB molecule was aligned and with a higher score alignment (28.062) when compared to the ligands model (27.042) (Fig. A.1).

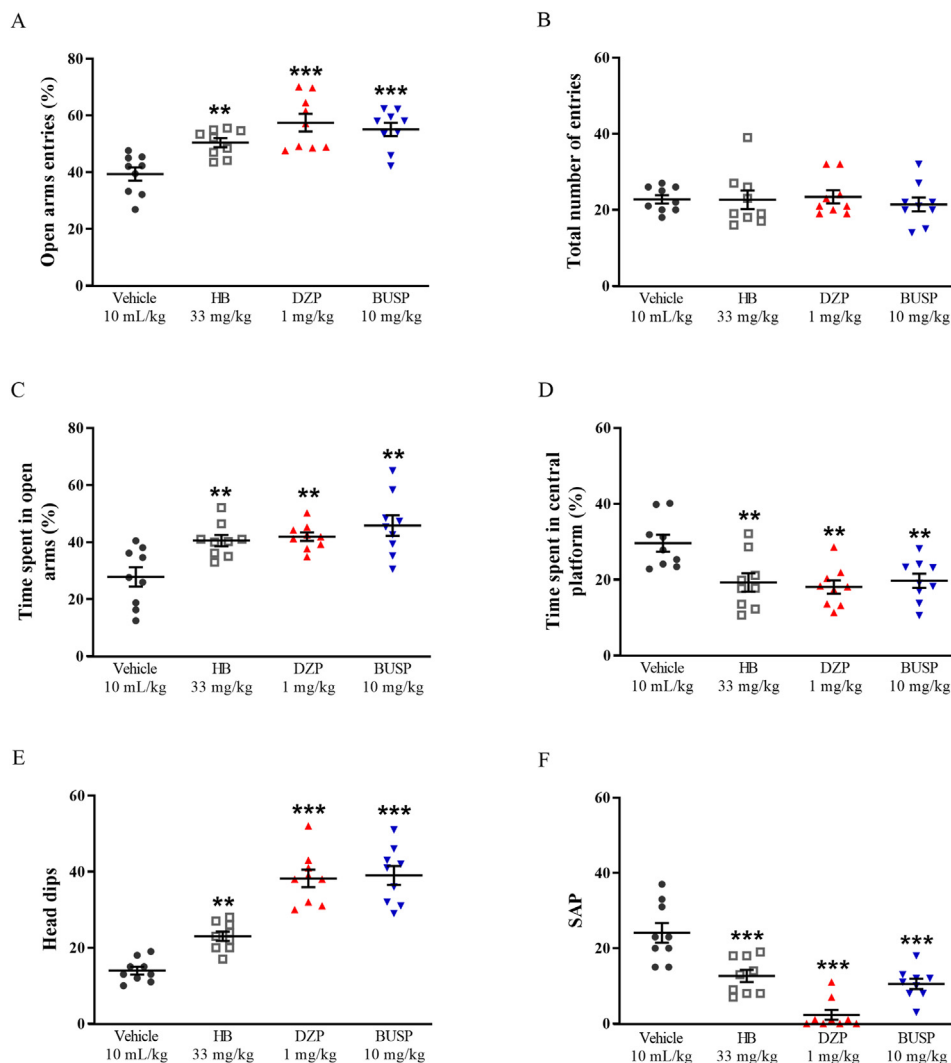A re-docking process with flumazenil complexed in $\alpha1\beta2\gamma2$

Fig. 2. Anxiolytic-like effects of hibalactone on elevated plus maze test. Results are expressed as mean ± SEM (n = 9). Diazepam and buspirone are used as positive controls. **p ≤ 0.01 and ***p ≤ 0.001 when compared with vehicle group (10% DMSO, 10 mL/kg) according to one-way ANOVA followed by Newnman-Keuls post hoc test. HB, hibalactone; DZP, diazepam; BUSP, buspirone; SAP, stretched-attend postures.

GABA$_A$ was done to validate the docking procedure. The 10-top ranked orientations of the ligand are presented in Fig. A.2. All the orientations presented heavy atoms RMSD <2 when compared with the crystallographic conformation. The docking protocol was thus able to reproduce the conformation and the binding mode of flumazenil, validating simulations with HB.

Overall structure of the reported α1β2γ2 GABA$_A$ receptor (PBD: 6D6U) is showed in Fig. 6a and the benzodiazepine site at α1-γ2 interface in the extracellular domain, where flumazenil interacts, is showed in Fig. 6b. Docking simulations revealed that the top-ranked HB and diazepam poses also bind at the same binding pocket. The ligands are nestled in an aromatic box formed mainly of Tyr-58, Phe-77, Phe-100, His-102, Tyr-160 and Tyr-210 residues. Fig. 6c and d are the 3D and 2D representations of the interactions of HB within the benzodiazepine binding site. Docked HB performs π−π interactions with Phe-77 and His-102; diazepam only with Phe-77 and flumazenil with Phe-100, Tyr-160, and Tyr-160. Hydrogen bond interactions were also observed for HB and diazepam with Ala-161, while flumazenil interacts with Thr-142. Predicted binding affinity showed that HB exhibited higher values compared to diazepam and flumazenil (Table 2). Poses of redocked flumazenil and diazepam as well as detailed ligand interaction diagrams are shown in Figs. A.3 and A.4, respectively.

4. Discussion

Hibalactone belongs to the lignan class which are molecules that show promise biological activities and have lead compounds for the development of new drugs (for review see Apers et al.[31]). In previous study, Oliveira et al.[10] showed the antinociceptive, anti-inflammatory and anxiolytic-like effects of *H. umbellata*, and after phytochemical screening these effects were attributed, at least in part, to the presence of HB. The present paper demonstrated that HB has significant anxiolytic-like effects on the light-dark box (LDB) and elevated plus maze (EPM) tests. The activity observed was reverted by flumazenil suggesting that activation of the benzodiazepine site of GABA$_A$ receptor contributes to anxiolytic-like effects of HB.

In the first phase of the study, we verified whether HB would present the same activity under the same conditions demonstrated
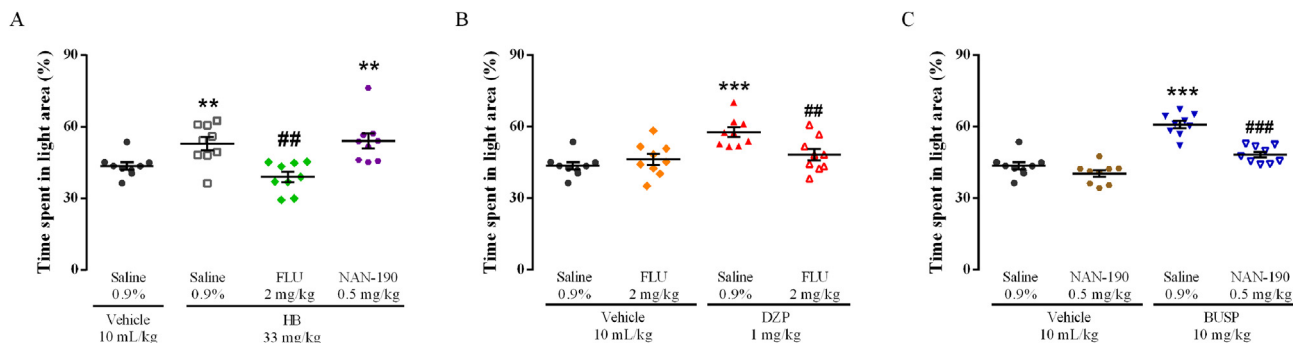
Fig. 3. Mechanism of action involved on anxiolytic-like effects of hibalactone in the percentage of time spent in light area on LDB test. (a) Effects of the pretreatment with 0.9% saline, flumazenil or NAN-190 on hibalactone effects on LDB. (b) Effects of the pretreatment with 0.9% saline or flumazenil on diazepam effects on LDB. (c) Effects of the pretreatment with 0.9% saline or NAN-190 on buspirone effects on LDB. The same control group (saline/vehicle) was used for all figures. Results are expressed as mean $\pm$ SEM (n = 9). **$p \leq 0.01$ and ***$p \leq 0.001$ when compared with saline/vehicle group by Student' T-Test. ##$p \leq 0.01$ and ###$p \leq 0.001$ when compared with saline/HB (a), saline/DZP (b) or saline/BUSP (c) group by Student' T-Test. HB, hibalactone; DZP, diazepam; BUSP, buspirone.
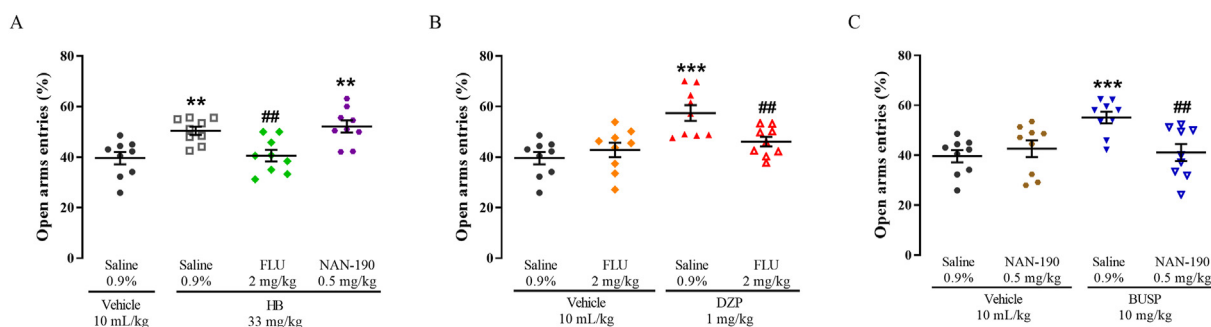

Fig. 4. Mechanism of action involved on anxiolytic-like effects of hibalactone in the percentage of open arms entries on EPM test. (a) Effects of the pretreatment with 0.9% saline, flumazenil or NAN-190 on hibalactone effects on EPM. (b) Effects of the pretreatment with 0.9% saline or flumazenil on diazepam effects on EPM. (c) Effects of the pretreatment with 0.9% saline or NAN-190 on buspirone effects on EPM. The same control group (saline/vehicle) was used for all figures. Results are expressed as mean $\pm$ SEM (n = 9). **$p \leq 0.01$ and ***$p \leq 0.001$ when compared with saline/vehicle group by Student' T-Test. ##$p \leq 0.01$ when compared with saline/HB (a), saline/DZP (b) or saline/BUSP (c) group by Student' T-Test. HB, hibalactone; DZP, diazepam; BUSP, buspirone.
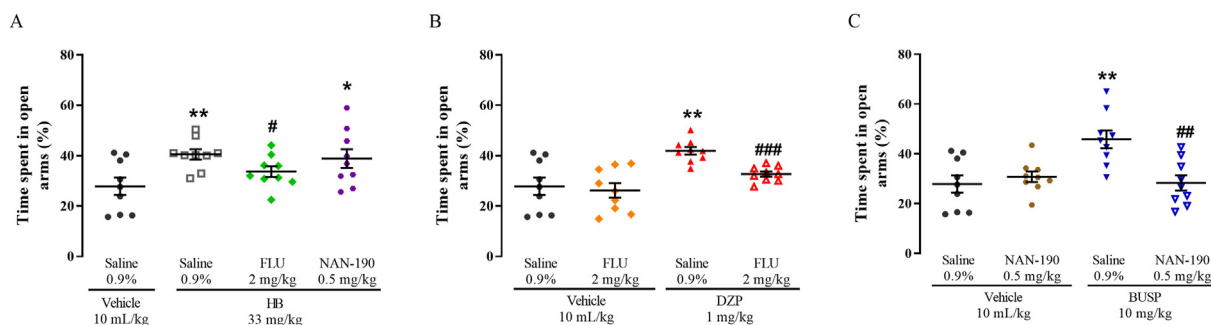

Fig. 5. Mechanism of action involved on anxiolytic-like effects of hibalactone in the percentage of time spent in the open arms on EPM test. (a) Effects of the pretreatment with 0.9% saline, flumazenil or NAN-190 on hibalactone effects on EPM. (b) Effects of the pretreatment with 0.9% saline or flumazenil on diazepam effects on EPM. (c) Effects of the pretreatment with 0.9% saline or NAN-190 on buspirone effects on EPM. The same control group (saline/vehicle) was used for all figures. Results are expressed as mean $\pm$ SEM (n = 9). *$p \leq 0.05$ and **$p \leq 0.01$ when compared with saline/vehicle group by Student' T-Test. #$p \leq 0.05$, ##$p \leq 0.01$ and ###$p \leq 0.001$ when compared with saline/HB (a), saline/DZP (b) or saline/BUSP (c) group by Student' T-Test. HB, hibalactone; DZP, diazepam; BUSP, buspirone.

Table 1
Hibalactone bioactivity prediction related to anxiolytic activity.

Pa	Pi	Activity	Web-server
0.751	0.006	Neurotransmitter uptake inhibitor	Pass
0.522	0.017	GABA aminotransferase inhibitor	
0.060	0.014	GABA uptake inhibitor	
0.411	0.180	Up-regulation of GABARAPL1 gene	DIGEP-Pred
-	–	α1β2γ2 GABA$_A$ receptor	SwissTargetPrediction

by Oliveira et al.[10] In this sense, the same dose of HB (33 mg/kg) administered orally was used and after 1 h the anxiolytic-like effect was evaluated in the LDB test, where it demonstrated the same effects by increase the percentage of time spent in the light area. Furthermore, the positive controls diazepam and buspirone also showed these effects. LDB test has been widely used as a tool in the investigation of anxiolytic-like effects in rodents, but for better screening it is recommended to perform more than one specific
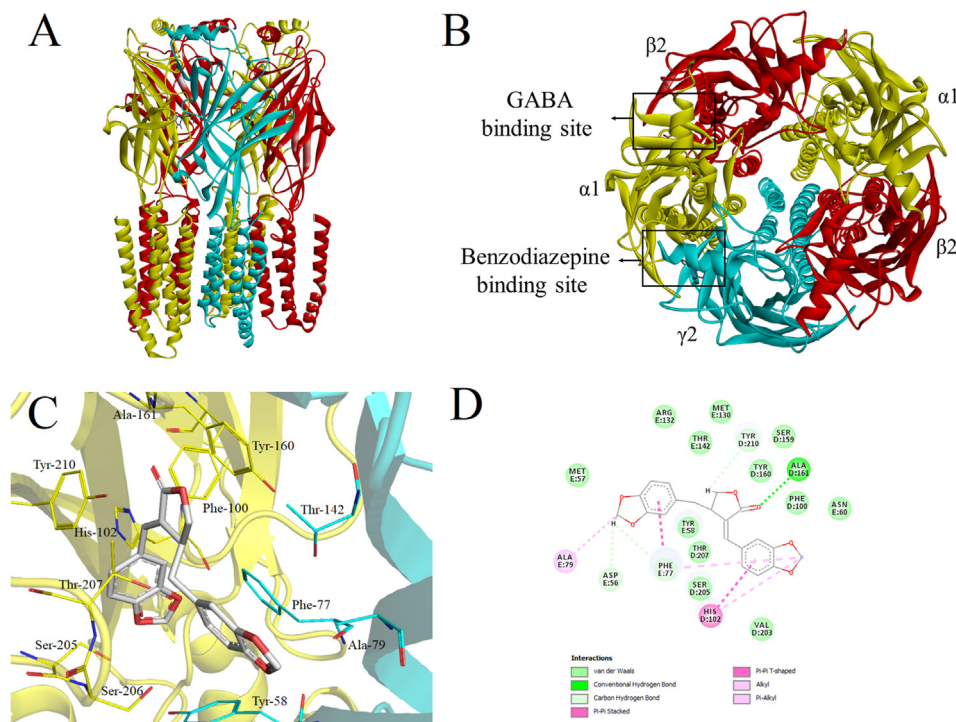
Fig. 6. (a) Side view of GABAA receptor isoform α1β2γ2. (b) Top view of the receptor: boxes indicate GABA binding site at β2–α1 interfaces and the benzodiazepine binding site at the α1–γ2 interface. (c) Docking 3D model of hibalactone (carbon atoms in gray) within benzodiazepine binding site, highlighting the main intermolecular interactions with GABAA residues (carbon atoms are color-coded in yellow and blue at the α1 and γ2 subunits, respectively). (d) 2D interaction diagram between the hibalactone and receptor residues.

Table 2
Predicted binding affinity, intermolecular energies and key interacting receptor residues of docked poses of hibalactone, diazepam and flumazenil.

Ligand	Affinity (kcal/mol)	*van der Waals* energy (kcal/mol)	Electrostatic energy (kcal/mol)	Key interacting residues
Hibalactone	−10.240	−28.624	−6.415	Phe-77, His-102 and Ala-161
Diazepam	−9.668	−29.010	−7.195	Phe-77 and Ala-161
Flumazenil	−9.336	−29.806	1.857	Phe-100, His-102, Thr-142, Tyr-160 and Tyr-210

test. Combining different tests is a way that an animal's instant emotional status can be assessed, through different tasks, can contribute to increasing the reliability and comprehensiveness of behavioral tests.[32] Hereupon, the effects of HB were assessed on EPM test.

EPM, as well as LDB, is based on the natural behavior of rodents, mainly, on the natural conflict between the drive to explore a new environment and the tendency to avoid a potentially dangerous area.[33] Treatment with HB and positive controls increased the percentage of entries and the time spent in open arms without changing the number total of entries, demonstrating that these treatments promote a preference for open arms of EPM with no alteration in the exploration of the apparatus. Furthermore, the percentage of time spent in the central platform was decreased. To improve the sensitivity of EPM, additional parameters known as ethological parameters were evaluated, evidenced by the analysis of the frequency of head dips and SAP. Drugs with an anxiolytic profile increase head dips frequency and reduced SAP frequency, whereas anxiogenic drugs do the opposite.[34] Therefore, treatment with HB and positive controls showed an anxiolytic profile as it promoted an increase of head dips suggesting further exploration of open arms, and a reduction of SAP that shows less interference from the potential risk induced by EPM.

In the second phase of the study, experimental assays were directed to understand which possible mechanisms of action are involved on the anxiolytic-like effect of HB. Anxiety disorder is a negative emotional state characterized by feelings of concern and apprehension, and accompanied by somatic, cognitive and behavioral manifestations resulting from complex gene-environment interactions.[35] There is growing evidence that, in anxiety disorders, occur a dysfunction in the modulation of brain circuits that regulate emotional responses to potentially threatening stimuli by changes in a diversity of neurotransmitter systems, especially in serotonergic and GABAergic pathways.[35,36]

Serotonergic neurons are implicated in altering appetite, energy, sleep, mood and cognitive function in anxiety disorders.[37] Evidence from the literature showed that most of the action of serotonin (5-HT) to moderate anxiety and stress occurs by inhibitory signaling via the 5-HT$_{1A}$ receptor.[38] Therefore, we investigated the participation of 5-HT$_{1A}$ receptor in observed activity with HB by pretreatment with the antagonist NAN-190. Our results showed that the anxiolytic-like effect of HB was not reversed by NAN-190 pretreatment, which allows us to suggest that this receptor does not participate directly in the mechanism of action of HB.

Most symptoms of anxiety disorders are mainly determined by the disbalance of excitatory and inhibitory inputs in CNS.[39]

GABAergic neurotransmission plays a fundamental role in inhibitory balance in normal and overexcited conditions, as in anxiety disorders, especially a rapid inhibition via GABA$_A$ receptors.[36,40] Herein, we investigated the participation of the benzodiazepine site of GABA$_A$ receptor in HB effects by pretreatment with the antagonist flumazenil. Results showed that the anxiolytic-like effect of HB was reverted by flumazenil, that is, the anxiolytic-like effects of HB could be mediated through activation of benzodiazepine site of GABA$_A$ receptor.

The biological activity of a compound is an intrinsic property dependent on its chemical structure.[41] Computational strategies may help elucidate the bioactivity of compounds, including the targets and mechanisms of action underlying some specific activities.[42,43] Predictions results by Pass, DIGEP-Pred and SwissTargetPrediction tools are compared with known experimental data for the studied compound using structure-activity relationships.[19–21]

Predictions revealed the potential of HB to increase GABAergic neurotransmission. Inhibition of GABA aminotransferase may reduce the degradation of GABA.[44] Extracts and phytochemicals of *Melissa officinalis* L., Lamiaceae, and *Valeriana officinalis* L., Caprifoliaceae, showed inhibitory activity against this enzyme.[45,46] Inhibition of GABA uptake is involved mainly with transport mechanisms, resulting in increased extracellular GABA levels,[47] which was also verified for the extract of *Passiflora incarnata* L., Passifloraceae.[48]

Gene expression changes are an important determinant of the drug effect on a cell.[20] The prediction of HB-induced gene expression profile demonstrated that it might influence the up-regulation of GABARAPL1. This gene contributes to GABA$_A$ receptors trafficking from the Golgi to the plasma membrane and the expression of the gene is related to a decrease in the internalization process of the receptor.[49]

Target prediction indicated that HB might interact with α1β2γ2 GABA$_A$ receptor. Heteromeric GABA$_A$ receptors are complexes of five subunits, usually composed of two α (α1−6), two β (β1−3) and a single γ (γ1−3), and the most common arrangement being α1β2γ2.[50] The GABA-binding site is found at the interfaces between β and α subunits, whereas the benzodiazepine-binding site is situated at the interface of α and γ subunits in the extracellular domain of the receptor (Fig. 6b).[51]

Pharmacophore modelling procedures were used first to identify the main features of the α1β2γ2 GABA$_A$ benzimidazole derivatives ligands reported in literature and to align these features with the ones of HB. Results indicated that the alignment of the aromatic rings and the hydrogen bond acceptor of HB structure allowed it to fit in the pharmacophore model and to fulfill the minimum requirements expected of ligands. Huang et al.[52] also showed the importance of these interactions in the pharmacophore model proposed to bind flavonoids at the benzodiazepine site in the α1γ2 pocket of GABA$_A$.

The high-resolution structure of the Human α1β2γ2 GABA$_A$ receptor, which illuminate atomic mechanisms of GABA and flumazenil recognition and features of the assembly of this heteromeric receptor, is described in the Protein Data Bank (PDB 6D6U).[28] Docking simulations with the receptor showed the allosteric interactions of HB at the benzodiazepine site, which occur mainly by aromatic and hydrogen bonding interactions. These interactions are in agreement with pharmacophore features of the most potent

ligands of this receptor. Moreover, HB interacts with key residues like His-102 and Phe-77, which were identified as being important, through mutagenesis studies, in the benzodiazepine and flumazenil binding.[50]

Thus, *in silico* findings support the experimental results and highlight that the activation of the benzodiazepine site of the GABA$_A$ receptor is involved in the anxiolytic-like effects of HB. It is known that classical GABAergic agonists can trigger considerable adverse effects, such as cognitive impairments, sedation and dependence,[53] therefore, it is crucial that the agonist action of HB on the GABA$_A$ receptor is further investigated regarding its pharmacokinetics and toxicodynamics aspects.

5. Conclusion

Hibalactone demonstrated anxiolytic-like effects involving the activation of the benzodiazepine site of the GABA$_A$ receptor, but not of 5-HT$_{1A}$ receptors. The experimental results were supported by *in silico* analysis, which mainly showed that hibalactone shares chemical features with GABA$_A$ ligands and may be a modulator at the benzodiazepine site of the α1β2γ2 isoform. Moreover, bioactivity predictions indicated that other mechanisms on GABA system might underlie the anxiolytic-effect of hibalactone, such as inhibition of degradation and uptake of GABA, which should be further investigated.

Declaration of competing interest

The authors declare no conflicts of interest.

List of Abbreviations

CNS	Central nervous system
DMSO	dimethyl sulfoxide
EPM	Elevated plus maze
GABA$_A$	Gamma-aminobutyric acid type A
GABARAPL1	GABA receptor-associated protein like 1
HB	Hibalactone
LDB	Light-dark box
NAN-190	1-(2-Methoxyphenyl)-4-[4-(2-phthalimido)butyl] piperazine hydrobromide
PDB	Protein data bank
RMSD	Root-mean-square deviation
SAP	Stretched-attend postures
5-HT1$_A$	5-hydroxytryptamine 1A receptor

Appendix A. Supplementary data

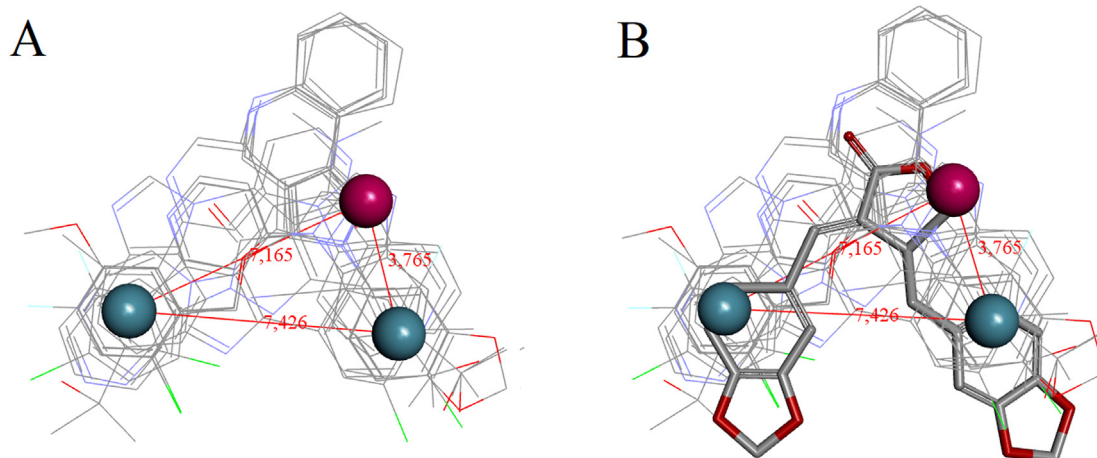Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2021.08.012.

Fig. A.1. (a) Pharmacophore model of the α1β2γ2 GABA$_A$ benzimidazole derivatives ligands. (b) Fitting of hibalactone to the model (b). Pharmacophore features are color-coded in blue for aromatic rings and pink for hydrogen-bond acceptors.
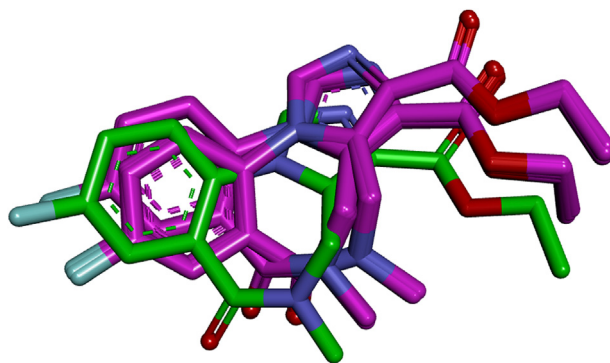

Fig. A.2. Superimposition of the crystallographic structure of flumazenil (carbon atoms in green) and the ten top-scored orientations (carbon atoms in purple) obtained by docking.
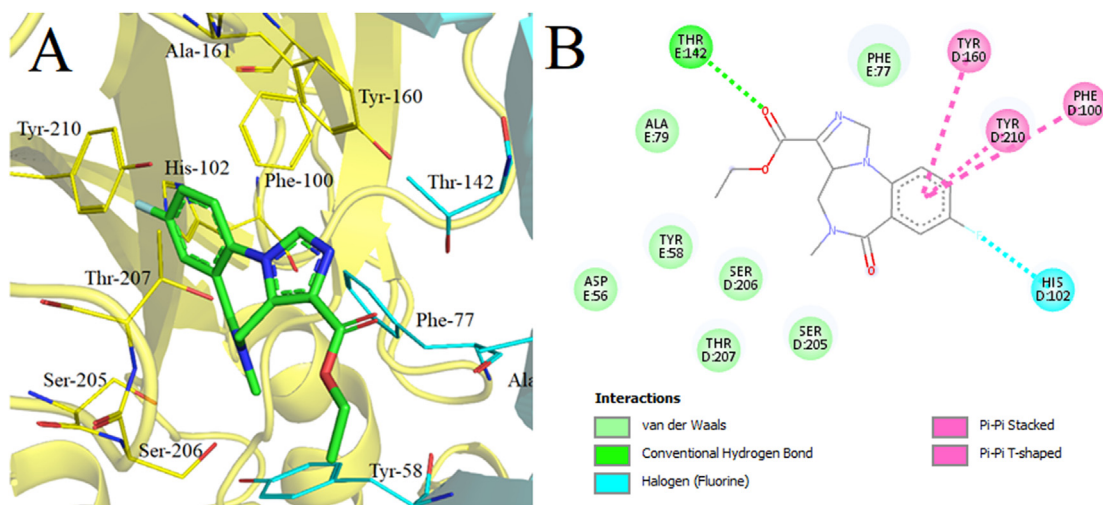

Fig. A.3. (a) Docking 3D model of flumazenil (carbon atoms in green) within benzodiazepine binding site, highlighting the main intermolecular interactions with GABAA residues (carbon atoms are color-coded in yellow and blue at the α1 and γ2 subunits, respectively). (b) 2D interaction diagram between the flumazenil and receptor residues.
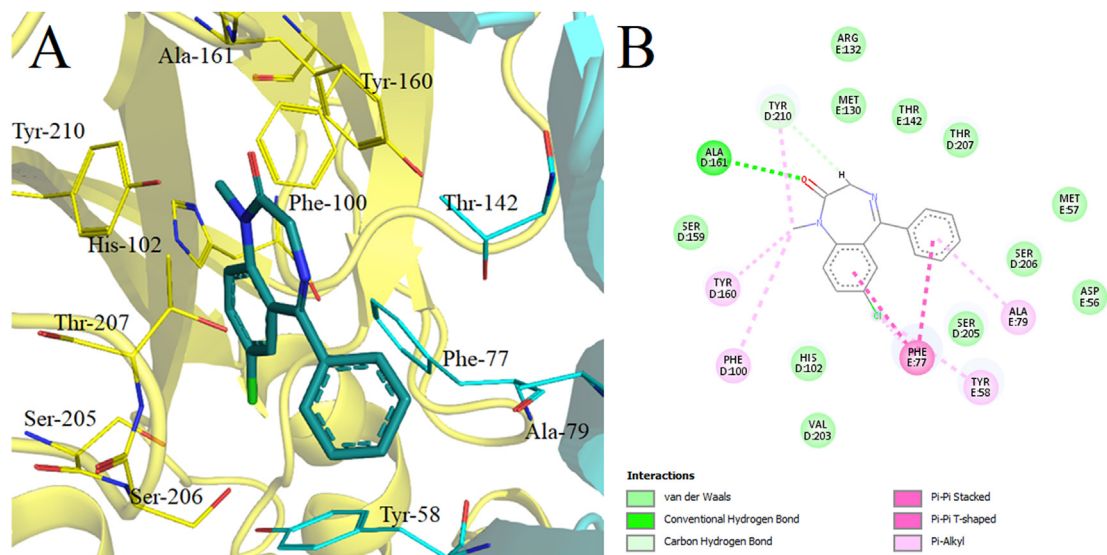
Fig. A.4. (a) Docking 3D model of diazepam (carbon atoms in blue) within benzodiazepine binding site, highlighting the main intermolecular interactions with GABAA residues (carbon atoms are color-coded in yellow and blue at the α1 and γ2 subunits, respectively). (b) 2D interaction diagram between the diazepam and receptor residues.

Appendix

Table A.I
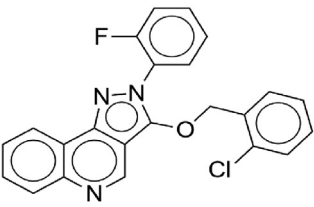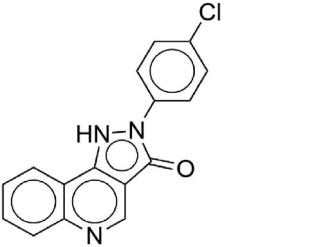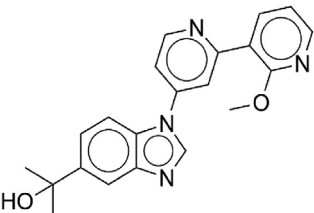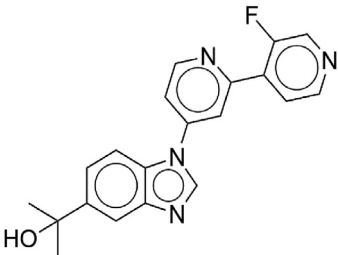Chemical structures and IC$_{50}$ values for α1β2γ2 GABA$_A$ benzimidazole derivatives ligands.
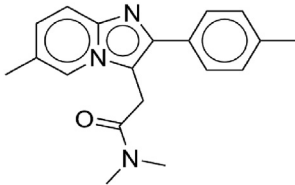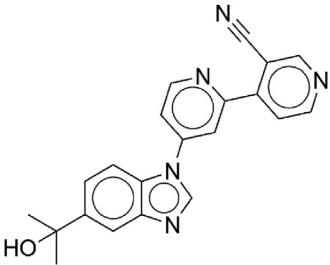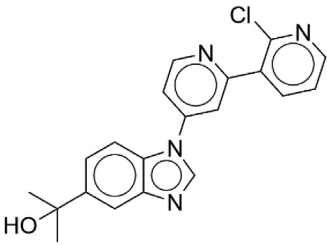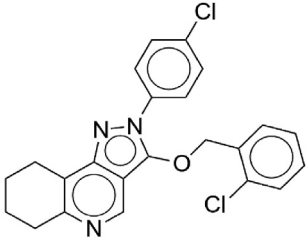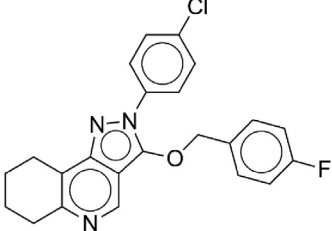
Structure	IC$_{50}$ (nm)	Reference
	1	Hintermann et al. (2011)
	1	Hintermann et al. (2011)
	1.2	Larsen et al. (2013)

Table A.I (*continued*)

Structure	IC$_{50}$ (nm)	Reference
	7.5	Larsen et al. (2013)
	14	Olivier et al. (1997)
	26	Larsen et al. (2013)
	38	Larsen et al. (2013)
	46	Hintermann et al. (2011)
	50	Hintermann et al. (2011)

Table A.I (*continued*)

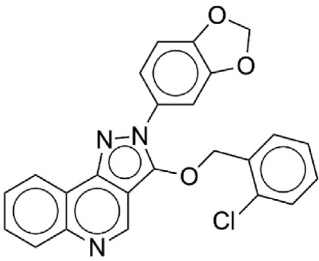| Structure	IC$_{50}$ (nm)	Reference
	50	Hintermann et al. (2011)

Table A.II

Hydrogen bond lengths and angles measured in the interactions of hibalactone, diazepam and flumazenil with α1β2γ2 GABAA receptor residues.

Ligand	Interacting residue	Hydrogen bond length	Hydrogen bond angle
Hibalactone	Ala-161	1.8 Å	150.16°
Diazepam	Ala-161	1.2 Å	168.00°
Flumazenil	Thr-142	3.1 Å	115.54°

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