European Journal of Pharmacology 873 (2020) 172962

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Full length article

Hops compounds modulatory effects and 6-prenylnaringenin dual mode of action on $GABA_A$ receptors

Ali Y. Benkherouf[a], Nora Logrén[b], Tamara Somborac[c], Maaria Kortesniemi[b], Sanna L. Soini[a], Baoru Yang[b], Outi M.H. Salo-Ahen[c], Oskar Laaksonen[b], Mikko Uusi-Oukari[a,*]

[a] Integrative Physiology and Pharmacology, Institute of Biomedicine, University of Turku, Finland
[b] Food Chemistry and Food Development, Department of Biochemistry, University of Turku, Finland
[c] Pharmaceutical Sciences Laboratory and Structural Bioinformatics Laboratory, Faculty of Science and Engineering, Åbo Akademi University, Turku, Finland

ARTICLE INFO

Keywords:
$GABA_A$ receptors
Allosteric modulation
Radioligand binding
Humulus lupulus
Molecular docking
Pharmacodynamics

ABSTRACT

Hops (*Humulus lupulus* L.), a major component of beer, contain potentially neuroactive compounds that made it useful in traditional medicine as a sleeping aid. The present study aims to investigate the individual components in hops acting as allosteric modulators in $GABA_A$ receptors and bring further insight into the mode of action behind the sedative properties of hops. GABA-potentiating effects were measured using [^{3}H]ethynylbicycloorthobenzoate (EBOB) radioligand binding assay in native $GABA_A$ receptors. Flumazenil sensitivity of GABA-potentiating effects, [^{3}H]Ro 15–4513, and [^{3}H]flunitrazepam binding assays were used to examine the binding to the classical benzodiazepines site. Humulone (alpha acid) and 6-prenylnaringenin (prenylflavonoid) were the most potent compounds displaying a modulatory activity at low micromolar concentrations. Humulone and 6-prenylnaringenin potentiated GABA-induced displacement of [^{3}H]EBOB binding in a concentration-dependent manner where the IC_{50} values for this potentiation in native $GABA_A$ receptors were 3.2 μM and 3.7 μM, respectively. Flumazenil had no significant effects on humulone- or 6-prenylnaringenin-induced displacement of [^{3}H]EBOB binding. [^{3}H]Ro 15–4513 and [^{3}H]flunitrazepam displacements were only minor with humulone but surprisingly prominent with 6-prenylnaringenin despite its flumazenil-insensitive modulatory activity. Thus, we applied molecular docking methods to investigate putative binding sites and poses of 6-prenylnaringenin at the $GABA_A$ receptor α1β2γ2 isoform. Radioligand binding and docking results suggest a dual mode of action by 6-prenylnaringenin on $GABA_A$ receptors where it may act as a positive allosteric modulator at α+β- binding interface as well as a null modulator at the flumazenil-sensitive α+γ2- binding interface.

1. Introduction

The female inflorescences of *Humulus lupulus* L., also known as hop cones, are a major component in beer brewing. These cones are rich in resins, essential oils, and polyphenols which exhibit antimicrobial properties and contribute to the beer aroma and distinctive bitter flavor (Karabín et al., 2016). Historically, hops have been medically used for insomnia and restlessness (Jackson, 1871), and the sedative effects of hops have been demonstrated in several studies using experimental models in rodents (Hansel et al., 1982; Wohlfart et al., 1983a, b). Zanoli et al. (2005) reported that alpha acids fraction from hops exhibits antidepressant properties in rats. Later in 2006, Schiller et al. showed that isolated hop oil, alpha acids and beta acids play a major role in the sedative effects of hops. These studies prompted further research to identify the neuroactive constituents in hops that are responsible for this activity and to reveal their mode of action on the central nervous system (CNS). Gamma-aminobutyric acid type A ($GABA_A$) receptors being the major site of fast synaptic inhibition in the CNS (Sieghart, 1995) are key potential targets for hops sedative compounds. Positive modulation of GABA-induced responses at $GABA_A$ receptors has been observed earlier by extracts of beer and hops (Aoshima et al., 2006; Sahin et al., 2016) as well as xanthohumol, the most abundant flavonoid in hops (Meissner and Häberlein, 2006). Recently, we examined the potential of several hops flavonoids in modulating the GABAergic activity and assessed their selectivity to $GABA_A$ receptor subtypes (Benkherouf et al., 2019). We identified potent prenylflavonoids in hops that positively modulate GABA-induced responses in native and αβγ/δ recombinant $GABA_A$ receptors at low micromolar concentrations. These GABAergic modulatory effects were not mediated via the high-affinity benzodiazepine binding site. Nevertheless, further

* Corresponding author. Kiinamyllynkatu 10, 20014, University of Turku, Finland.
E-mail address: mikko.uusi-oukari@utu.fi (M. Uusi-Oukari).

https://doi.org/10.1016/j.ejphar.2020.172962
Received 2 October 2019; Received in revised form 17 December 2019; Accepted 24 January 2020
Available online 27 January 2020

Table 1
Isolated fractions from semipreparative liquid chromatography and the tentative identification of compounds.

Fraction	Compound	Retention time (min) [b]	UV–vis λ_{max} (nm)	QTOF-MS (m/z)						Basis of identification [a]
				$[M + H]^+$ measured	$[M + H]^+$ calculated	Error (ppm)	$[M - H]^-$ measured	$[M - H]^-$ calculated	Error (ppm)	
F1	Xanthohumol	7.57	195/365	355.1527	355.1540	3.7	353.1387	353.1394	3.5	UV–vis, MS, NMR
F2	Cohumulone	11.17	196/236/284/322	349.2000	349.2010	2.8	347.1907	347.1864	12.4	UV–vis, std, MS
F3	Humulone	12.43	196/236/285/323	363.2150	363.2166	4.4	361.2007	362.2020	2.7	UV–vis, MS, NMR
F4	Adhumulone	12.96	196/226/284/322	363.2153	363.2166	3.6	361.2067	362.2020	9.8	UV–vis, std, MS, NMR
F5 [c]	Adprehumulone/prehumulone, postlupulone	14.70	192/236/282/322	377.2307/387.2509	377.2323/387.2530	4.2/5.4	375.2199/385.2375	375.2177/385.2384	5.9/2.4	UV–vis, MS, NMR
F6	Colupulone	15.44	195/228/276/334	401.2669	401.2686	4.6	399.2606	399.2541	16.3	UV–vis, std, MS, NMR
F7	Lupulone/adlupulone	16.05	–	–	–	–	–	–	–	
F7.1 [b]	Lupulone	11.45	193/229/276/333	415.2825	415.2843	4.3	413.2760	413.2697	15.1	UV–vis, std, MS, NMR
F7.2 [b]	Adlupulone	12.25	192/236/285/332	415.2819	415.2843	5.8	413.2733	413.2697	8.9	UV–vis, std, MS

$[M - H]^-$ ion 361.2004). Based on NMR, F5 contains also humulone but the presence of postlupulone could not be verified.

[a] Tentative identification of the fraction based on the standard mixture (ICE-3), UV–vis spectra, MS analyses and literature.

[b] Retention times in the semipreparative liquid chromatography. Fractions 7.1 and 7.2 isolated using the second LC gradient.

[c] Adprehumulone and prehumulone have same masses. F5 contain traces of humulone and/or adhumulone (measured $[M + H]+$ ion 363.2149 and $[M - H]-$ ion 361.2004). Based on NMR, F5 contains also humulone but the presence of postlupulone could not be verified.

potential neuroactive compounds from hops need to be isolated and characterized in terms of their pharmacological activity.

The present study aims to investigate the individual components in hops acting as allosteric modulators in $GABA_A$ receptors and bring further insight into the mode of action behind hops sedative properties. GABA-potentiating effects were measured using [^{3}H]ethynylbicycloorthobenzoate (EBOB) radioligand binding assay in native $GABA_A$ receptors. Flumazenil sensitivity of GABA-potentiating effects, displacement of [^{3}H]Ro 15–4513, and [^{3}H]flunitrazepam binding were used to examine the binding to the classical benzodiazepines site. Furthermore, we implied molecular docking methods to investigate putative binding sites and poses of 6-prenylnaringenin at the $GABA_A$ receptor α1β2γ2 isoform.

2. Materials and methods

2.1. Reagents

The radioligands [propyl-2,3-^{3}H]EBOB (48 Ci/mmol), [7,9-^{3}H]Ro 15–4513 (28 Ci/mmol), and [methyl-^{3}H]flunitrazepam (85.2 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). Diazepam, GABA and picrotoxin were from Sigma Chemical Co. (St. Louis, MO, USA). Flumazenil (Ro 15–1788) was from Tocris Bioscience (Bristol, UK). Humulone standard was purchased from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany). ICE-3 standard containing a mixture of α-acids cohumulone (13.88%) and N + adhumulone (30.76%), and β-acids colupulone (13.44%) and N + adlupulone (10.84%), was from Labor Veritas AG (Zurich, Switzerland). HPLC grade solvents (methanol, acetonitrile) and formic acid used in the sample preparation were from Sigma-Aldrich Sigma Chemical Co. (St. Louis, MO, USA). Methanol-d_4 (CD_3OD, 99.8 %D) containing tetramethylsilane (TMS) as an internal standard was from VWR Chemicals (Leuwen, Belgium). Chloroform-*d* ($CDCl_3$, 99.96 %D) containing TMS was from Eurisotop (St. Aubin Cedex, France).

2.2. Animals

A total of 20 native male Sprague-Dawley rats (age: 11–13 weeks) were purchased from the Central Animal Laboratory of the University of Turku (Turku, Finland). The rats were euthanized by guillotine decapitation; their fore/midbrain and cerebellum were dissected, frozen on dry ice and stored at −70 °C. All procedures were in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Turku (license number: 25715/2018).

2.3. Plant material preparation and extraction

Xanthohumol, α-acids and β-acids were extracted from commercial hops pellets (Citra, purchased from homebrew supply store Lappo Ltd., Piikkiö, Finland). Two grams of hops was extracted two consecutive times with 10 mL of acidified methanol (MeOH:HCOOH, 99:1, v/v). Sample was shaken vigorously for 30 min at room temperature and then centrifuged at 1500 *g* for 5 min at room temperature. The two collected extracts were combined, complemented to a total volume of 20 mL with the solvent and finally filtered using 0.2 μm polytetrafluoroethylene (PTFE) filters. Sample was stored at −20 °C for further HPLC analysis.

2.4. Preparative HPLC

The compounds from the hops extract were isolated using a Shimadzu (Shimadzu, Kyoto, Japan) semipreparative HPLC system consisting of a SIL-20A autosampler, an LC-20AB pump, a CTO-10AC column oven, an SPD-20A UV-detector, and a DGU-20A5 degasser. The column was an Aeris PEPTIDE XB-C18 (Phenomenex, Torrance, CA), 250 mm × 10.0 mm i.d., 5 μm, equipped with a 10 mm × 10 mm i.d. guard column of the same material (Phenomenex, Torrance, CA). The chromatograms were recorded at 332 nm. Injection volume was 80 μL. Elution was carried out with a binary solvent system with 0.1% formic acid in Milli-Q water as solvent A and 0.1% formic acid in acetonitrile as B with the flow rate of 4 mL/min. The fractions were collected with two separate gradient programs. The first program for fractions 1–6 (Table 1): 0–3 min, B 2–80%; 3–10 min, B 80%; 10–12 min, B 80–100%; 12–16.5 min, 100%; 16.5–17.5 min, 100–2%. The second eluent program was used to separate the seventh fraction due to insufficient separation in the first eluent program; 0–3 min, B 2–90%; 3–13 min, B 90%; 13–14 min, B 90–2%. Separated fractions were first dried using a rotary evaporator at +30 °C and then the flasks were washed twice with 1 mL of methanol and the methanol was removed under nitrogen flow in a heat block at +30 °C. Resulting dry fractions were weighed and dissolved in DMSO at 10 mM concentrations. Fraction F5 was also freeze-dried prior to the NMR analysis.

2.5. Identification of the compounds

Seven of the eight isolated compounds (F1–F4, F6–F7.2, Table 1) were identified based on references (ICE-3, Labor Veritas AG, Zurich, Switzerland), UV–vis spectra, mass spectra, and literature (Zhang et al., 2004; Češlová et al., 2009; Intelmann et al., 2009; Prencipe et al., 2014) (Table 1). Exact masses were measured using the high-resolution Bruker Impact II(TM) UHR-QqTOF (Bruker Daltonik GmbH, Bremen, Germany) mass spectrometer in positive and negative MS/MS modes using electrospray ionization (ESI). The compounds were separated using a Bruker Elute UHPLC equipped with a HPG1300 pump and a diode array detector (DAD, 190–800 nm) with the same separation conditions as previously described (chapter 2.4). The column used was a Kinetex 2.6 μm C18 100 Å (100 × 4.6 mm) column (Phenomenex Inc., Torrance, CA). The parameters for ESI + ionization were: capillary voltage of 4.5 kV, the end plate offset 500 V, nebulizer gas (N_2) at a pressure of 4.8 bar, the drying gas (N_2) flow and temperature at 12.0 L/min and 350 °C, respectively. The mass range was *m/z* 20–1000. Calibration was carried out by injecting 10 mM sodium formate with 400 μL/min flow rate from a direct infusion syringe pump to the six-port valve for high-accuracy mass experiments in the HPC mode. The instrument was controlled and the data was handled with the Compass DataAnalysis software 4.4 (Bruker Daltonik).

^{1}H and ^{13}C NMR spectra were obtained for further identification of F5. The isolate F5 (2.17 mg) was dissolved in 600 μL of chloroform-*d* and analyzed with standard 1D and 2D experiments (^{1}H, ^{13}C, COSY, HSQC-ed, HMBC) at 298 K using 500 MHz Bruker Avance-III NMR system (Fällanden, Switzerland) equipped with a SmartProbe (operating at 500 MHz and 125 MHz for ^{1}H and ^{13}C, respectively). The spectra were phase- and baseline-corrected with Bruker TopSpin software.

2.6. Investigation of commercial hops compounds

In addition to the fractioned compounds from Citra hops pellets, we tested several compounds present in hops that have potential neuroactive properties. These include 2-methyl-3-buten-2-ol (2M3B), purchased from Sigma Chemical Co. (St. Louis, MO, USA) as well as 6-prenylnaringenin (6PN) and linalool, purchased from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany).

2.7. [^{3}H]EBOB binding assay

Rat fore/midbrain and cerebellar membrane preparation for binding studies was modified from Squires and Saederup (2000) and essentially performed as described in Benkherouf et al. (2019). Frozen membranes were thawed, washed once by centrifugation at 10,000 *g* for 10 min and resuspension in assay buffer (50 mM Tris-HCl, 120 mM NaCl, pH 7.4), and resuspended finally in the same buffer. Triplicate samples of rat brain membranes were incubated at room temperature

with shaking for 2 h in assay buffer with 1 nM or 2 nM [^{3}H]EBOB and different concentrations of hops compounds (30 nM - 30 μM) in the absence or presence of 2 μM GABA in a total volume of 400 μL. Non-specific binding was determined with 100 μM picrotoxin. The incubation was terminated by filtration of the samples with a Brandel Cell Harvester (model M-24, Gaithersburg, MD, USA) onto Whatman GF/B glass fiber filters (Whatman International Ltd., Maidstone, UK). The samples were rinsed three times with 5 mL of ice-cold 10 mM Tris-HCl, pH 7.4. Air-dried filters were immersed in 3 mL of Optiphase HiSafe 3 scintillation fluid (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) and radioactivity determined in Hidex 600 SL liquid scintillation counter (Hidex, Turku, Finland).

2.8. [^{3}H]Ro 15–4513 and [^{3}H]flunitrazepam binding assay

[^{3}H]Ro 15–4513 and [^{3}H]flunitrazepam binding assays were modified from Uusi-Oukari and Korpi (1992). Quadruplicate samples were incubated on ice at 4 °C with shaking for 1 h in assay buffer (see above) with 2 nM [^{3}H]Ro 15–4513 or 1 nM [^{3}H]flunitrazepam in the absence and presence of the test compound in a total volume of 300 μL. Non-specific binding was determined in the presence of 10 μM flumazenil. The incubation was terminated by filtration of the samples onto Whatman GF/B glass fiber filters and washing them twice with 5 mL of ice-cold 10 mM Tris-HCl, pH 7.4 in [^{3}H]Ro 15–4513 binding or 10 mM Tris-HCl, pH 7.4, 120 mM NaCl in [^{3}H]flunitrazepam binding as similarly described for [^{3}H]EBOB binding.

2.9. Molecular docking

The Glide XP docking algorithm (Friesner et al., 2004, 2006) and the Induced Fit docking (IFD) protocol (Sherman et al., 2006a, 2006b) of Schrödinger's Maestro Suite (release 2019-1) were used to dock the hops compounds into the putative binding sites at the α1+γ2- and α1+β2- subunit interfaces. Additionally, the $GABA_AR$ benzodiazepine site antagonist flumazenil and the positive allosteric $GABA_AR$ modulator pyrazoloquinoline 2-p-methoxyphenylpyrazolo[4,3-c]quinolin-3(5H)-one (CGS 9895) (Varagic et al., 2013) were docked as reference compounds at these sites, respectively (see the detailed description of the docking protocol in the Supplementary Material). Binding energies of the docked compounds were evaluated with the Prime/MMGBSA tool of Maestro (Lyne et al., 2006; Sastry et al., 2013; Walters et al., 1998). Visual analysis of the docking results was carried out with Maestro and PyMOL (version 2.3; Schrödinger, LLC).

2.10. Statistics

GraphPad Prism 7 software (GraphPad, San Diego, CA, USA) was used in statistical testing and curve fitting of the radioligand displacement data. Inhibition data were fit to the sigmoidal dose-response equation with a variable Hill Slope:

Y = Bottom + (Top-Bottom)/(1 + ((X^HillSlope)/(IC_{50}^HillSlope)))where Y is the percentage of control binding, Bottom = 0 when non-specific binding is subtracted from all binding values, Top is the maximum value, and X is the test compound concentration. Nonlinear least squares regression analysis was used for estimation of the half maximal inhibitory concentration (IC_{50}) for [^{3}H]EBOB non-competitive binding which are presented as means ± S.E.M. IC_{50} value obtained from [^{3}H]flunitrazepam displacement curve (competitive binding) was converted to Ki value according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973):

$$Ki = IC_{50} /(1 + ([L]/K_d))$$

where Ki = inhibitory constant, [L] = concentration of [^{3}H]flunitrazepam (1 nM) and K_d = dissociation constant of [^{3}H]flunitrazepam (1.1 nM) (Bosmann et al., 1978).

The statistical comparisons were performed with unpaired *t*-test and one-way ANOVA followed by Dunnett's *post hoc* test. P-values of less than 0.05 were considered significant.

3. Results

3.1. Isolation HPLC, and MS analysis

Seven major peaks in the ethanol extract of the hops pellets were first identified as xanthohumol (F1), cohumulone (F2), humulone (F3), adhumulone (F4), colupulone (F6), lupulone (F7.1), adlupulone (7.2) based on the commercial standard, retention times in literature (Zhang et al., 2004; Češlová et al., 2009; Intelmann et al., 2009; Prencipe et al., 2014) and UV–vis spectra (approx. λ maximum at 365 for xanthohumol, 320 nm for humulones and 330 nm for lupulones) (fractions F1–F4 and F6–F7.2 in Table 1). Due to their similar structures, n- and adhumulones and corresponding lupulones do not easily separate in the LC chromatogram and additional preparative LC gradient was required for these hops components. Peak F5 detected between adhumulone and colupulone with a similar UV–vis spectra to the humulones was also selected for the fraction collection. Compounds in the ethanol fraction were further studied with the QTOF-MS and exact masses for measured ions and calculated ions for the compounds are shown in Table 1. While other peaks contained essentially the measured $[M+H]^+$ or $[M-H]^-$ ions that corresponded to the calculated ions, the fraction F5 contained ions for at least four different compounds indicating presence of adprehumulone and/or prehumulone (both have the same molecular mass) and postlupulone, as well as, adhumulone (or humulone) (Zhang et al., 2004; Češlová et al., 2009).

3.2. NMR spectroscopy

NMR spectra were used to further verify the identification of compounds in the fractions (Table 1, Supplementary Table S1) and to assess their purity. F1 and F3 consisted of one compound each (xanthohumol and humulone, respectively); however, the composition of F2 was a very complex mixture of up to five α-acid-type compounds. The relative proportions based on the integral values for the 3-OH resonance peaks were 1.0 (19.00 ppm, attributed to cohumulone), 0.5 (18.82 ppm), 6.2 (18.72 ppm), 4.3 (18.64 ppm) and 2.7 (18.63 ppm). The primary compound in F4 was assigned as adhumulone (approx. 85%), with a minor proportion of humulone. With 1D and 2D NMR, the F5 was confirmed as a mixture of humulone and two other α-acids, of which adprehumulone was assigned as the main compound (Smith and Wilson, 1992). The structure of the hexanoyl side chain corresponding to adprehumulone was ultimately determined by COSY and ^{1}H–^{13}C correlations observed in the multiplicity-edited HSQC experiment revealing the otherwise obstructed methylene resonances (Supplementary Fig. S2). In F5, the proton resonances at 0.93 ppm (2 d/dd, J = 6.6, 1.3 Hz) with ^{13}C-correlations to 22.5 ppm (HSQC) and 37.9 ppm (HMBC) could be assigned to prehumulone or similar structure (Smith and Wilson, 1992), however, the other overlapping signals in the spectra of F5 obstructed its verification. The relative proportion of adprehumulone: humulone: prehumulone was 2:1:1. The presence of postlupulone in F5 was not verified with NMR. F6 was confirmed to be colupulone based on the 3-OH resonances (Supplementary Table S1) and contains trace amounts of linalool and lipids. The NMR sample concentrations of F7.1 and F7.2 were very low considering the lower sensitivity of NMR to MS, therefore their interpretation was challenging. Nevertheless, lupulone presence was confirmed in F7.1.

3.3. Allosteric modulation of [^{3}H]EBOB binding in native $GABA_A$ receptors

The modulation of [^{3}H]EBOB binding from rat forebrain and cerebellar membranes was first investigated with 30 μM concentration of hops compounds in the presence of 2 μM GABA (Fig. 1). All tested

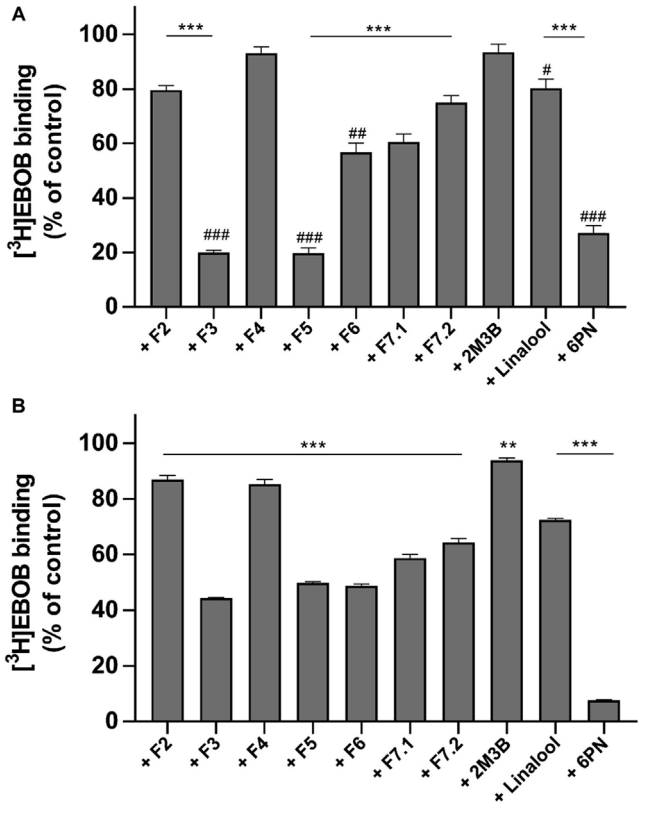

Fig. 1. Modulation of [^{3}H]EBOB (1 nM) binding in rat forebrain (A) and cerebellar (B) membranes by hops compounds (30 μM) in the presence of 2 μM GABA. Control is the maximal [^{3}H]EBOB binding in the presence of 2 μM GABA alone. The values represent means ± S.E.M., n = 3–5, measured in triplicates. ***P < 0.001, **P < 0.01 for the significance of difference from control binding (one-way ANOVA followed by Dunnett's *post hoc* test). $^{###}$P < 0.001, $^{##}$P < 0.01, $^{#}$P < 0.05 for the statistical difference from the corresponding [^{3}H]EBOB binding in cerebellum (unpaired *t*-test).

compounds, except adhumulone (F4) and 2M3B in forebrain, displayed evident enhancements in GABA-induced [^{3}H]EBOB displacement. The observed range of [^{3}H]EBOB displacement was 19.7–80% in rat forebrain and 6.2–92.4% in cerebellum. Humulone (F3), F5 and 6-prenylnaringenin were the most efficient compounds in forebrain as they displaced 80.0%, 80.3% and 72.9% of [^{3}H]EBOB binding, respectively (P < 0.001, one-way ANOVA followed by Dunnett's *post hoc* test). The activity of these compounds at 30 μM were significantly different in cerebellum (P < 0.001, unpaired *t*-test) where humulone (F3), F5 and 6-prenylnaringenin displaced 55.6%, 50.2% and 92.4% of GABA-induced [^{3}H]EBOB binding, respectively (P < 0.001, one-way ANOVA followed by Dunnett's *post hoc* test).

Humulone (F3), F5, lupulone (7.1), methyl-3-buten-2-ol (2M3B), linalool, and 6-prenylnaringenin (6PN) at 30 μM were selected to examine their direct effects on GABA-free [^{3}H]EBOB binding in native $GABA_A$ receptors. [^{3}H]EBOB binding was insensitive to displacement in the absence of GABA except for 2M3B, which displaced 14.3% of specific [^{3}H]EBOB binding (P < 0.01, one-way ANOVA followed by Dunnett's *post hoc* test) (Fig. 2).

The effects of compounds displaying high enhancements in GABA-induced [^{3}H]EBOB displacement were further assessed in a concentration series to determine their inhibitory potency on the radioligand binding in the presence of 2 μM GABA. 6-prenylnaringenin, humulone (F3), and F5 showed a concentration-dependent potentiation of GABA-induced [^{3}H]EBOB displacement in rat forebrain membranes at low micromolar concentrations (Fig. 3). For instance, 6-prenylnaringenin and humulone (F3) at 1 μM concentration displaced 35.2 ± 0.5% (P < 0.001) and 39.6 ± 1.6% (P < 0.001) of [^{3}H]EBOB binding,

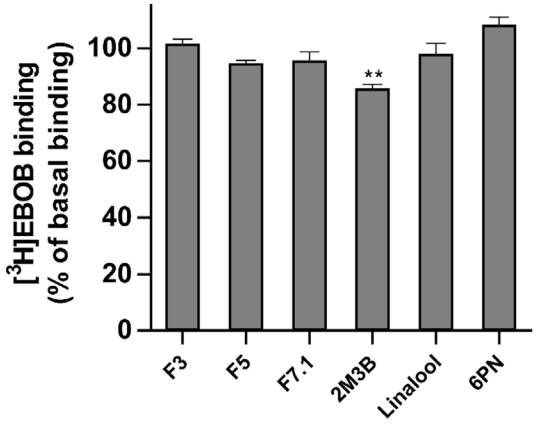

Fig. 2. Displacement of [^{3}H]EBOB (1 nM) binding in rat forebrain membranes by selected active hops compounds (30 μM) in the absence of GABA. The values represent means ± S.E.M., n = 3, measured in triplicates. **P < 0.01 for the significance of difference from control (one-way ANOVA followed by Dunnett's *post hoc* test).

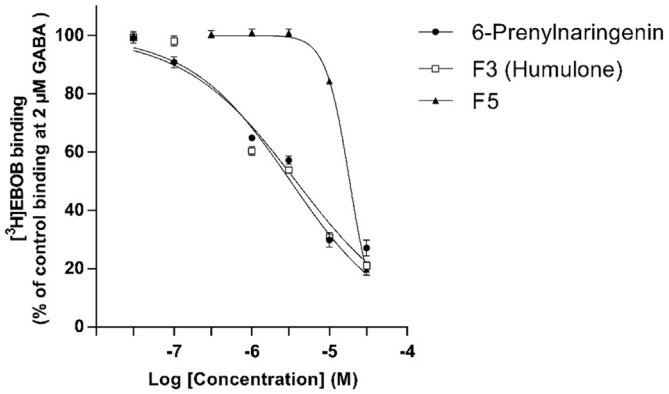

Fig. 3. Displacement curves of [^{3}H]EBOB (1 nM) binding in rat forebrain membranes with 5–6 concentrations of 6-prenylnaringenin humulone (F3), and F5 in the presence of GABA (2 μM). Control is the maximal [^{3}H]EBOB binding in the presence of 2 μM GABA alone. The values represent means ± S.E.M., n = 3, measured in triplicates.

while F5 effect was absent at 3 μM and below. The calculated IC_{50} values of 6-prenylnaringenin, humulone (F3) were 3.7 ± 0.4 μM, 3.2 ± 0.4 μM, and 18.2 ± 0.4 μM, respectively.

3.4. Flumazenil sensitivity of allosteric modulation of [^{3}H]EBOB binding

We examined whether the flumazenil-sensitive benzodiazepine site is essential for the low micromolar allosteric modulation exhibited by hops compounds. Despite the evident antagonizing effect of flumazenil on the allosteric modulation by diazepam (**P < 0.01), flumazenil failed to inhibit the enhancement in GABA-induced [^{3}H]EBOB displacement produced by 6-prenylnaringenin, humulone (F3), F5 and lupulone (7.1) at native $GABA_A$ receptors (Fig. 4). In fact, a slight but statistically significant further enhancement in [^{3}H]EBOB displacement was detected as a result of flumazenil addition.

3.5. Competitive displacement of [^{3}H]flunitrazepam and [^{3}H]Ro 15–4513 binding to the benzodiazepine site of $GABA_A$ receptors

We further tested the effect of hops compounds at 30 μM concentration on the binding of [^{3}H]flunitrazepam (Fig. 5A), a positive allosteric modulator, and the binding of [^{3}H]Ro 15–4513 (Fig. 5B), a partial inverse agonist of $GABA_A$ receptor benzodiazepine site. Non-specific binding was determined using flumazenil (10 μM) for both

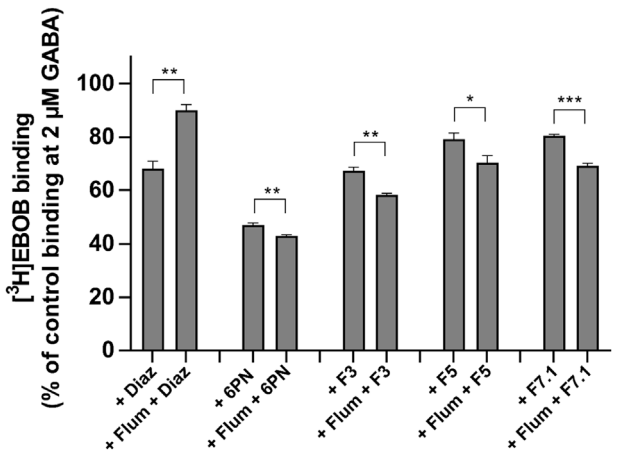

Fig. 4. Modulation of [^{3}H]EBOB (1 nM) binding in rat forebrain membranes by diazepam (Diaz, 0.1 μM), 6-prenylnaringenin (6PN, 5 μM), humulone (F3, 1 μM), lupulone (F7.1, 20 μM) and F5 (10 μM) in the presence of GABA (2 μM) and flumazenil (Flum, 2 μM). Control is the maximal [^{3}H]EBOB binding in the presence of 2 μM GABA alone. The values represent means ± S.E.M., n = 3–5, measured in triplicates. ***P < 0.001, **P < 0.01, *P < 0.05 for the significance of difference from the corresponding control binding (unpaired *t*-test).

radioligands. 6-prenylnaringenin showed significant effects on the radioligands as it displaced 47.1 ± 4.1% (P < 0.001) and 59.4 ± 0.48% (P < 0.001) of [^{3}H]flunitrazepam and [^{3}H]Ro 15–4513 binding, respectively. 6-prenylnaringenin showed a concentration-dependent displacement of [^{3}H]flunitrazepam (Fig. 5C) where the calculated Ki value of 6-prenylnaringenin according to the Cheng-Prusoff equation was 16.5 μM, which is substantially higher than the reported Ki value for diazepam (14 nM) in [^{3}H]flunitrazepam binding assay (Forbes et al., 1990). On the other hand, only a minor displacement of [^{3}H]flunitrazepam was observed with cohumulone (F2) (15.6 ± 2.0%, P < 0.01) and humulone (F3) (17.0 ± 4.8%, P < 0.01) at 30 μM. To a lesser extent, cohumulone (F2) and humulone (F3) displaced 9.2 ± 2.3% (P < 0.01) and 7.5 ± 2.0% (P < 0.01) of [^{3}H]Ro 15–4513 binding at the same concentration, respectively.

3.6. Molecular docking

Molecular docking studies were carried out to investigate putative binding sites and poses of the hops prenylflavonoids at the $GABA_AR$ α1β2γ2 isoform (PDB ID: 6D6U; Zhu et al., 2018). Flumazenil and CGS 9895 were used as reference compounds in terms of the Prime/MMGBSA energies and the docking scores in their respective binding pockets (Fig. 6A and B). Docking results for 6-prenylnaringenin are shown in Table 2. In general, hops prenylflavonoids show comparable binding energies with the reference compounds at the studied binding sites (Supplementary Table S2). Putative binding poses of 6-prenylnaringenin at the α1 + γ2- and α1 + β2- sites are shown in Fig. 6C and D, respectively. Poses of the other prenylflavonoids and the reference compounds as well as detailed ligand interaction diagrams of all compounds are shown in Supplementary Figs. S3–S8.

4. Discussion

4.1. Alpha acids

The major components of hops resins are alpha acids, which contribute to 5–13% of the weight of dried hops (Almaguer et al., 2014). These play a major role in beer brewing due to their foam stabilizing and antibacterial properties. Alpha acids are partially isomerized during the boiling process of brewing to form iso-α-acids bringing the

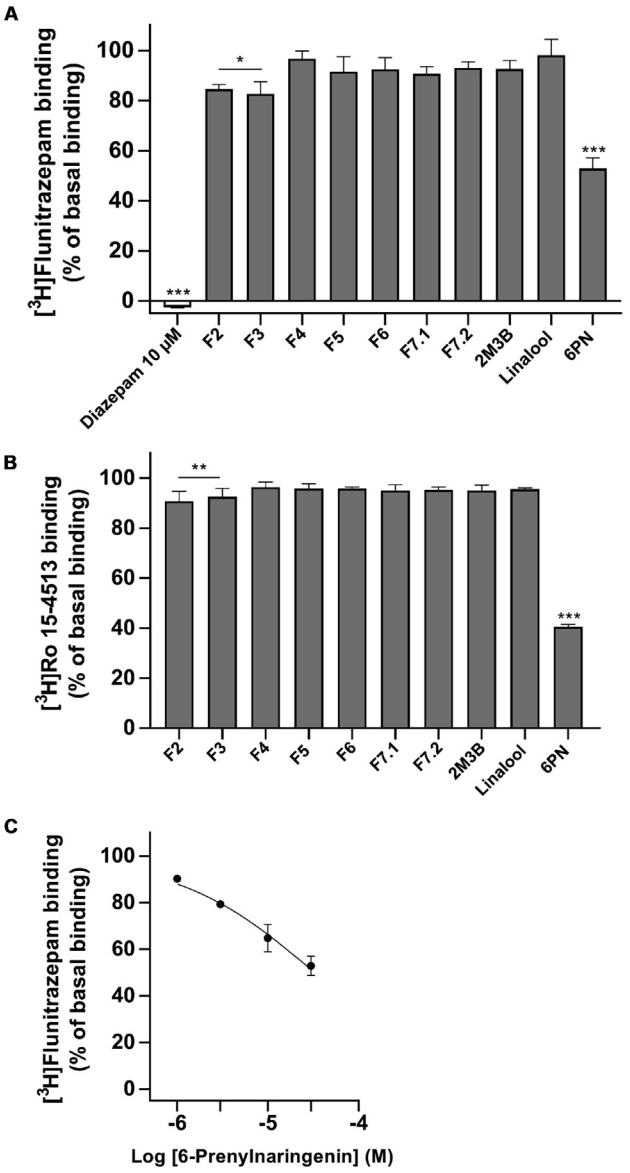

Fig. 5. Modulation of [^{3}H]flunitrazepam (1 nM) (A) and [^{3}H]Ro 15–4513 (2 nM) (B) binding in rat forebrain membranes by 30 μM of hops compounds. The values represent means ± S.E.M., n = 3, measured in quadruplicate. ***P < 0.001, **P < 0.01, *P < 0.05, significantly different from the corresponding basal radioligand binding (one-way ANOVA followed by Dunnett's *post hoc* test). Displacement curve of [^{3}H]flunitrazepam (1 nM) (C) binding in rat forebrain membranes with 4 concentrations of 6-prenylnaringenin. The values represent means ± S.E.M., n = 6, measured in triplicates.

bitter characteristic of beer (Clarke and Hildebrand, 1965; Verzele et al., 1965). *In vivo* studies showed that alpha acid extracts exhibit sedative activity by enhancing pentobarbital-induced sleeping time in rats but with no impact on locomotor activity (Zanoli et al., 2005). Later in 2006, Schiller et al. showed that alpha acid extracts at higher doses were able to enhance ketamine-induced sleeping time in mice as well as reduce locomotor activity. The positive modulation of GABA-induced displacement of [^{3}H]EBOB binding by the isolated alpha acid components: humulone, cohumulone and adhumulone brings a pharmacological basis for the sedative properties observed in earlier studies. Nevertheless, due to the weak modulatory effects observed with cohumulone and adhumulone, their probable role in sedation is minor compared to humulone itself.

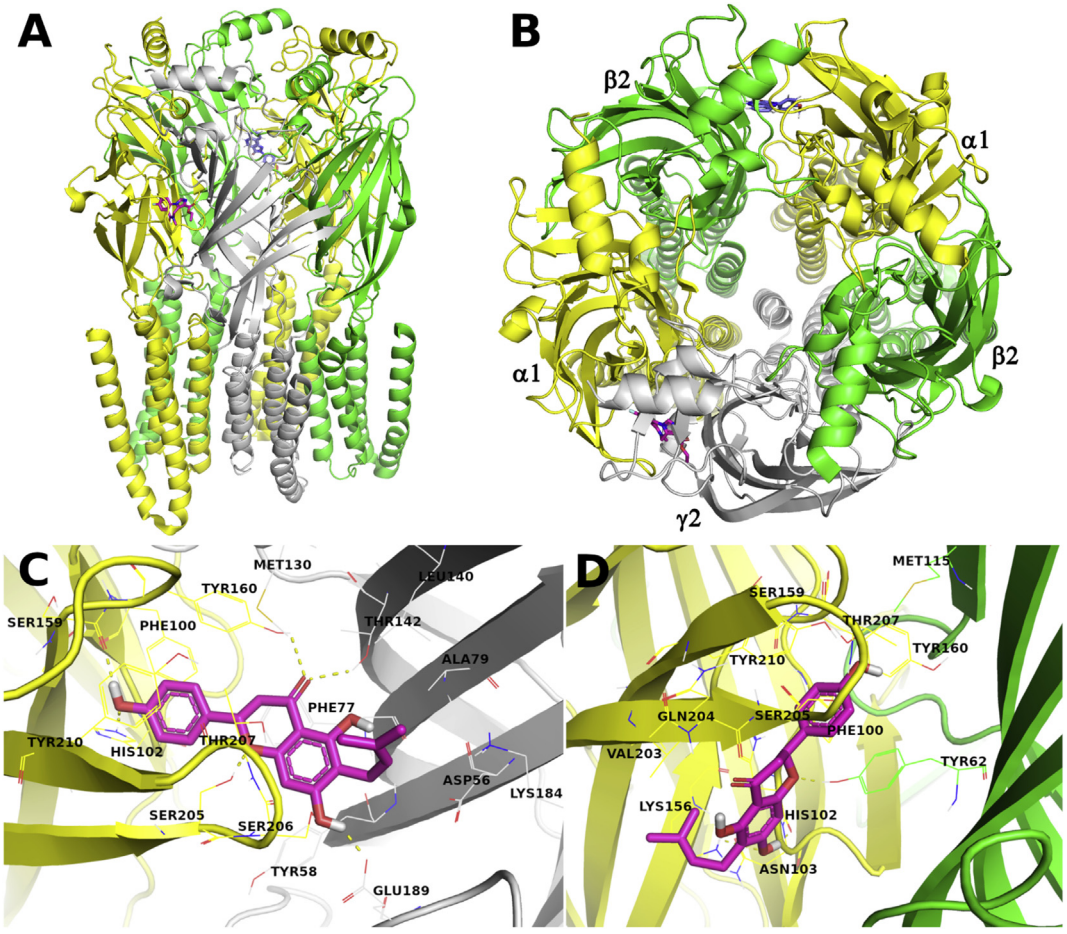

Fig. 6. α1 + γ2- and α1 + β2- binding sites of the $GABA_A$ receptor isoform α1β2γ2 (PDB ID: 6D6U; cartoon presentation; α1 subunit in bright yellow, γ2 in grey, β2 in green). A) Side view of the receptor. B) Top view of the receptor. A) - B) Reference compounds in their respective binding sites: experimental pose of flumazenil (magenta sticks); docked pose of CGS 9895 (marine blue sticks). C) Docked pose of 6-prenylnaringenin (magenta sticks) at the α1 + γ2- site. and D) at the α1 + β2- site. Interacting residues are shown in lines and labeled; non-polar hydrogen atoms are omitted for clarity; atom color code: carbon – magenta, nitrogen – blue, oxygen – red, fluorine – light blue, sulfur – dark yellow; hydrogen – white). Polar interactions are shown as dotted yellow lines.

4.2. Beta acids

Beta acids, on the other hand, represent 3–8% of the weight of dried hops and mainly contains lupulone, colupulone and adlupulone (Van Cleemput et al., 2009; Almaguer et al., 2014). The positive modulatory activity exhibited by beta acids are in line with their sedative properties (Schiller et al., 2006). However, their probable role in sedation is minor compared to alpha acids due to the relative lower potency according to our *in vitro* results as well as the findings of *in vivo* studies (Schiller et al., 2006). Despite the anti-depressant activity reported by Zanoli et al. (2007) in rats, lower doses of beta acids, in fact, led to a reduction in pentobarbital-induced sleeping time as well as an increase in motor performance. Moreover, electrophysiological recordings of beta acids in cerebellar granule cells (CGC) showed a flumazenil-insensitive negative modulation of GABA-induced currents at 120 μM (Zanoli et al., 2007) in contrast to the positive modulation observed in our [^{3}H]EBOB binding assay at 30 μM. These discrepancies do not exclude the role of beta acids in modulating $GABA_A$ receptors but suggests further investigation on the probable dose-dependent biphasic behavior of its components.

4.3. 2-Methyl-3-butene-2-ol (2M3B)

During the storage of hops at room temperature, alpha and beta acids undergo oxidative degradation to form 2-methyl-3-butene-2-ol, a C5 alcohol that is found in trace amounts of hops extracts (< 0.01%) (Hansel et al., 1980, 1982). This compound displayed sedative properties as well as narcosis in rodents, but in doses beyond its natural presence in hops (200–800 mg/kg.bw) (Hansel et al., 1980; Wohlfart et al., 1983a, 1983b). These finding correlate with the minor or non-existent effects observed in our binding results.

4.4. Linalool

Linalool, a monoterpene alcohol, is a major component of hop essential oils detected in amounts up to 150 mg/kg of total hop oil (Štěrba et al., 2015) adding a floral and fruity character to beer (Almaguer et al., 2014; Peacock and Deinzer, 1981). Linalool was found to display hypnotic, sedative and anxiolytic properties in mice without impairing motor performance (Linck et al., 2010; Guzman-Gutierrez et al., 2012). Electrophysiology studies with *Xenopus* oocytes and HEK293 cells showed enhancements in GABA-induced responses by linalool at 1–2 mM (Hossain et al., 2002; Aoshima et al., 2006; Milanos et al., 2017). Interestingly, our radioligand binding results revealed a low micromolar linalool sensitivity to native $GABA_A$ receptors where its modulatory effects can be observed at concentrations down to 30 μM. This may further support the role of $GABA_A$ receptor modulation in the sedative and anxiolytic properties of linalool.

4.4.1. 6-Prenylnaringenin

6-prenylnaringenin is an isomer of the prenylflavonoid 8-prenylnaringenin and considered as a weak phytoestrogen (< 1/100 of 8PN) (Milligan et al., 1999) as well as potent T-type calcium channel

Table 2
Docking results for 6-prenylnaringenin at the α1 + γ2-and α1 + β2- binding pockets of the experimental structure of $GABA_AR$ α1β2γ2 subtype (PDB ID: 6D6U).

Ligand	Binding site α1 + γ2-			Binding site α1 + β2-			
	Glide XP score (kcal/mol)	MMGBSA ΔG bind (kcal/mol)	Key interacting receptor residues from subunits $\alpha_1\gamma_2$[d]	Glide XP score (kcal/mol)[e]	IFD Score[f]	MMGBSA ΔG bind (kcal/mol)[g]	Key interacting receptor residues from subunits $\alpha_1\beta_2$[h]
6-prenyl-naringenin (6PN)	−8.255	−71.13	His102, Ser159, Tyr160, Ser205, Ser206, Tyr210/Thr142, Glu189	−4.356	−3002.55	−29.95/-67.11	Phe100, His102, Lys156, Gln204, Ser205, Tyr210/Tyr62
Flumazenil	−9.001[a]/-6.494[b]	−57.95[b]/-64.71[b]	Phe100, His102 Ala161, Thr207, Tyr210/ Thr142	na	na	na	na
CGS 9895	na[c]	na	na	−2.246	−2992.31	−23.33/-69.64	His102, Ser206, Tyr210/Tyr62

[a] The top-ranked (XP score) docking pose that is flipped ~180° along the longest axis of the molecule compared to the experimental pose (Supplementary Figs. S3 and S4).
[b] Docking pose similar to the experimental pose (cf. for the initial experimental pose the MMGBSA energy was −57.52 kcal/mol – only the added hydrogen atoms of the receptor had been minimized while the heavy atoms were restrained).
[c] Not applicable.
[d] His102 protonated at epsilon nitrogen (HIE102).
[e] Non-optimal (rigid) binding site conformation.
[f] Induced fit docking composite score = GlideScore + 0.05 × PrimeEnergy.
[g] Prime/MMGBSA free energy of binding calculated for the respective Glide XP/induced fit docked poses.
[h] His102 protonated at delta nitrogen (HIS102).

blocker (Sekiguchi et al., 2018). The precursor of 6-prenylnaringenin and other hops iso-flavanones is desmethylxanthohumol (DMX) (Stevens et al., 1999). During brewing, and due to the low stability of DMX in aqueous solutions (Miles and Main, 1985), it undergoes spontaneous isomerization into 6-prenylnaringenin and to a lesser extent into 8-prenylnaringenin (Hansel and Schulz, 1988; Chadwick et al., 2006). Despite being found in low concentrations in hops < 0.01% (Almaguer et al., 2014), the compound received considerable attention for its promising bioactivity. These include antifungal (Mizobuchi and Sato, 1984), antiosteoporotic (Effenberger et al., 2005), antiproliferative (Delmulle et al., 2006, 2008; Venturelli et al., 2018; Bartmanska et al., 2018) as well as pain-relieving properties (Sekiguchi et al., 2018; Du Nguyen et al., 2018).

6-prenylnaringenin appears to be the most potent hops prenylflavonoid acting as a positive allosteric modulator with an IC_{50} of 3.7 ± 0.4 μM for GABA-induced [^{3}H]EBOB displacement in native forebrain $GABA_A$ receptors. The corresponding IC_{50} for xanthohumol, isoxanthohumol and 8-prenylnaringenin were 29.7 ± 0.8 μM, 11.6 ± 0.7 μM and 7.3 ± 0.4 μM, respectively (Benkherouf et al., 2019). Despite the structural similarities between 6-prenylnaringenin and its isomer 8-prenylnaringenin, our radioligand binding results show clear differences in terms of their modulatory potency, direct agonist effects on $GABA_A$ receptors and selectivity to the classical benzodiazepine binding site. For example, the more potent modulator, 6-prenylnaringenin, was unable to induce changes in GABA-free [^{3}H]EBOB binding at 30 μM, while 8-prenylnaringenin at the same concentration displaced 51% of [^{3}H]EBOB binding in the absence of GABA (Benkherouf et al., 2019). Moreover, 6-prenylnaringenin was more efficient in displacing [^{3}H]Ro 15–4513 binding to the classical benzodiazepine binding site in native $GABA_A$ receptors.

In a study by Sekiguchi et al. (2018), 6-prenylnaringenin was found to cross the blood-brain barrier (BBB) with no impact on locomotor activity with open field and rotarod tests in mice. The concentrations detected 10 and 30 min after i.p. administration in mice brain (30 mg/kg) were 1.95 μM and 2.34 μM, respectively. Both concentrations are sufficient to modulate GABA-induced responses in native $GABA_A$ receptors *in vitro*. Given the fact that the isomer 8-prenylnaringenin was found to induce panicolytic effects with elevated T-maze test in rats (Bagatin et al., 2014), further studies are needed to confirm the behavioral effects of 6-prenylnaringenin in anxiolysis and sleep-promoting activity.

4.4.2. $GABA_A$ receptor subtype selectivity and putative 6-prenylnaringenin binding site

According to our results, flumazenil led to a slight potentiation in GABA-induced [^{3}H]EBOB displacement confirming its intrinsic partial agonist property reported in α6-containing $GABA_A$ receptors (Hadingham et al., 1996) and some animal models (Dantzer and Perio, 1982; Kaijima et al., 1983; Vellucci and Webster, 1983; Belzung et al., 2000). Nevertheless, the positive modulation of 6-prenylnaringenin, humulone and lupulone in [^{3}H]EBOB binding was insensitive to flumazenil antagonism suggesting that these compounds exert their modulatory activity through a site other than the classical benzodiazepine binding pocket.

The displacement of GABA-induced [^{3}H]EBOB binding by 6-prenylnaringenin is higher in cerebellar than in forebrain membranes. Moreover, an earlier study using recombinant $GABA_A$ receptors expressed in HEK293 cells showed that hops prenylflavonoids: 8-prenylnaringenin and isoxanthohumol, modulate the activity of α6β3δ receptors with higher potency compared to α1β3γ2 (Benkherouf et al., 2019). Since δ subunit exists as a major α6β2/3δ subtype in CGC (Quirk et al., 1995; Jechlinger et al., 1998; Pirker et al., 2000; Pöltl et al., 2003), our results suggest a higher modulatory potency of 6-prenylnaringenin in the benzodiazepine-insensitive δ-containing $GABA_A$ receptors.

Our molecular docking results at $GABA_A$ receptor α1β2γ2 isoform

show that the most favorable free energy of binding is predicted for 6-prenylnaringenin at both α1 + β2- and α1 + γ2- interfaces (Table 2). The best docking poses were selected based on the estimated free energy of binding (Prime/MMGBSA), Glide XP docking score or IFD Score and visual assessment. Based on our earlier findings (Benkherouf et al., 2019), the allosteric modulation exhibited by hops prenylflavonoids was not dependent on γ or δ subunit suggesting a major role of α + β-interface as a binding site in $GABA_A$ receptor pentameric complex. Point mutations at the α1 subunit of the mouse receptor have indicated that Tyr209 (Tyr210 in human receptor) was the key residue involved in the mode of action of our reference compound CGS 9895 that acts as a positive allosteric modulator at the α1 + β2- site (Varagic et al., 2013; Maldifassi et al., 2016; Ramerstorfer et al., 2011). Consistently, Tyr210 is interacting with CGS 9895 and all hops prenylflavonoids in our binding site model.

Docking results at α1 + γ2- binding interface of $GABA_A$ receptor α1β2γ2 isoform revealed structural similarities and comparable binding energies between flumazenil and 6-prenylnaringenin. In addition, the best ligand poses were considered to be those that exhibited many of the favorable interactions reported for flavonoids (Renard et al., 1999; Huang et al., 2001), such as aromatic stacking and hydrogen bond interactions. 6-prenylnaringenin is predicted to form aromatic stacking interactions and hydrogen bonds with the key binding site residues that also participate in flumazenil and benzodiazepine agonist binding (Table 2; Buhr et al., 1996; Jursky et al., 2000; Richter et al., 2012; Zhu et al., 2018). Of note, the docking pose of 6-prenylnaringenin at the α1 + γ2- site (Fig. 6C) is fairly consistent with the flavonoid pharmacophore model reported by Huang et al. (2001). In radioligand binding assays, 6-prenylnaringenin significantly displaced both [^{3}H]flunitrazepam and [^{3}H]Ro 15–4513 despite the lack of involvement of the classical benzodiazepine site in its allosteric activity. Hence, we suggest that 6-prenylnaringenin may act as a null modulator at the classical benzodiazepine site where it binds to the same binding site as flumazenil at the α1 + γ2- subunit interface.

5. Conclusions

We presented individual components in hops that potentiate GABA-induced displacement of [^{3}H]EBOB binding in native $GABA_A$ receptors bringing further understanding of the possible mechanism of hops sedative properties. Humulone (alpha acid) and 6-prenylnaringenin (prenylflavonoid) were the most potent compounds displaying modulatory activities at low micromolar concentrations. These modulations appear to be not mediated by the flumazenil-sensitive benzodiazepine binding site. Nevertheless, radioligand binding and molecular docking results suggest a dual mode of action by 6-prenylnaringenin on $GABA_A$ receptors where it may act as a positive allosteric modulator at α + β-interface as well as a null modulator at the flumazenil-sensitive α + γ2-interface. Since hops intake occurs mainly through beer consumption in humans, further electrophysiological and behavioral studies are needed to confirm the synergic effects of the reported modulators with the presence and absence of alcohol.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This project was financially supported by The Finnish Foundation for Alcohol Studies (A.B., M.U.-O.), Turku University Foundation (S.L.S.) and Turku Drug Research Doctoral programme (A.B.). The Sigrid Jusélius Foundation, Biocenter Finland Bioinformatics and Drug Discovery and Chemical Biology networks, CSC IT Center for Science, Joe, Pentti and Tor Borg Memorial Fund and Prof. Mark Johnson and Dr. Jukka Lehtonen are gratefully acknowledged for the excellent computational infrastructure at the Åbo Akademi University (O.M.H.S.-A.)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2020.172962.

References

Almaguer, C., Schönberger, C., Gastl, M., Arendt, E.K., Becker, T., 2014. Humulus lupulus – a story that begs to be told. A review. J. Inst. Brew. 120, 289–314. https://doi.org/10.1002/jib.160.

Aoshima, H., Takeda, K., Okita, Y., Hossain, S.J., Koda, H., Kiso, Y., 2006. Effects of beer and hop on ionotropic gamma-aminobutyric acid receptors. J. Agric. Food Chem. 54, 2514–2519. https://doi.org/10.1021/jf051562a.

Bagatin, M.C., Tozatti, C.S., Abiko, L.A., Yamazaki, D.A., Silva, P.R., Perego, L.M., Audi, E.A., Seixas, F.A., Basso, E.A., Gauze Gde, F., 2014. Molecular docking and panicolytic effect of 8-prenylnaringenin in the elevated T-maze. Chem. Pharm. Bull. (Tokyo) 62, 1231–1237. https://doi.org/10.1248/cpb.c14-00569.

Bartmanska, A., Tronina, T., Poplonski, J., Milczarek, M., Filip-Psurska, B., Wietrzyk, J., 2018. Highly cancer selective antiproliferative activity of natural prenylated flavonoids. Molecules 23, E2922. https://doi.org/10.3390/molecules23112922.

Belzung, C., Le Guisquet, A.M., Crestani, F., 2000. Flumazenil induces benzodiazepine partial agonist-like effects in BALB/c but not C57BL/6 mice. Psychopharmacology (Berlin) 148, 24–32. https://doi.org/10.1007/s002130050021.

Benkherouf, A.Y., Soini, S.L., Stompor, M., Uusi-Oukari, M., 2019. Positive allosteric modulation of native and recombinant GABA A receptors by hops prenylflavonoids. Eur. J. Pharmacol. 852, 34–41. https://doi.org/10.1016/j.ejphar.2019.02.034.

Bosmann, H.B., Penney, D.P., Case, K.R., DiStefano, P., Averill, K., 1978. Diazepam receptor: specific binding of [^{3}H]diazepam and [^{3}H]flunitrazepam to rat brain subfractions. FEBS Lett. 87, 199–202. https://doi.org/10.1016/0014-5793(78)80331-7.

Buhr, A., Baur, R., Malherbe, P., Sigel, E., 1996. Point mutations of the alpha 1 beta 2 gamma 2 gamma-aminobutyric acid(A) receptor affecting modulation of the channel by ligands of the benzodiazepine binding site. Mol. Pharmacol. 49, 1080–1084. https://www.ncbi.nlm.nih.gov/pubmed/8649346.

Češlová, L., Holčapek, M., Fidler, M., Drštičková, J., Lísa, M., 2009. Characterization of prenylflavonoids and hop bitter acids in various classes of Czech beers and hop extracts using high-performance liquid chromatography–mass spectrometry. J. Chromatogr. A, Adv. Separ. Methods Food Anal. 1216, 7249–7257. https://doi.org/10.1016/j.chroma.2009.09.022.

Chadwick, L.R., Pauli, G.F., Farnsworth, N.R., 2006. The pharmacognosy of Humulus lupulus L. (hops) with an emphasis on estrogenic properties. Phytomedicine 13, 119–131. https://doi.org/10.1016%2Fj.phymed.2004.07.006.

Clarke, B.J., Hildebrand, R.P., 1965. The isomerization of humulone. J. Inst. Brew. 71, 26–36. https://doi.org/10.1002/j.2050-0416.1965.tb02019.x.

Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108. https://doi.org/10.1016/0006-2952(73)90196-2.

Dantzer, R., Perio, A., 1982. Behavioural evidence for partial agonist properties of RO 15-1788, a benzodiazepine receptor antagonist. Eur. J. Pharmacol. 81, 655–658. https://doi.org/10.1016/0014-2999(82)90355-7.

Delmulle, L., Vanden Berghe, T., Keukeleire, D.D., Vandenabeele, P., 2008. Treatment of PC-3 and DU145 prostate cancer cells by prenylflavonoids from hop (Humulus lupulus L.) induces a caspase-independent form of cell death. Phytother Res. 22, 197–203. https://doi.org/10.1002/ptr.2286.

Delmulle, L., Bellahcene, A., Dhooge, W., Comhaire, F., Roelens, F., Huvaere, K., Heyerick, A., Castronovo, V., De Keukeleire, D., 2006. Anti-proliferative properties of prenylated flavonoids from hops (Humulus lupulus L.) in human prostate cancer cell lines. Phytomedicine 13, 732–734. https://doi.org/10.1016/j.phymed.2006.01.001.

Du Nguyen, H., Okada, T., Kitamura, S., Yamaoka, S., Horaguchi, Y., Kasanami, Y., Sekiguchi, F., Tsubota, M., Yoshida, S., Nishikawa, H., Kawabata, A., Toyooka, N., 2018. Design and synthesis of novel anti-hyperalgesic agents based on 6-prenylnaringenin as the T-type calcium channel blockers. Bioorg. Med. Chem. 26, 4410–4427. https://doi.org/10.1016/j.bmc.2018.07.023.

Effenberger, K.E., Johnsen, S.A., Monroe, D.G., Spelsberg, T.C., Westendorf, J.J., 2005. Regulation of osteoblastic phenotype and gene expression by hop-derived phytoestrogens. J. Steroid Biochem. Mol. Biol. 96, 387–399. https://doi.org/10.1016/j.jsbmb.2005.04.038.

Forbes, I.T., Johnson, C.N., Jones, G.E., Loudon, J., Nicholass, J.M., Thompson, M., Upton, N., 1990. Synthesis and evaluation of a series of aryl[e]fused pyrazolo[4,3-c] pyridines with potential anxiolytic activity. J. Med. Chem. 33, 2640–2645. https://doi.org/10.1021/jm00171a046.

Friesner, R.A., Murphy, R.B., Repasky, M.P., Frye, L.L., Greenwood, J.R., Halgren, T.A., Sanschagrin, P.C., Mainz, D.T., 2006. Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. J. Med. Chem. 49, 6177–6196. https://doi.org/10.1021/jm051256o.

Friesner, R.A., Banks, J.L., Murphy, R.B., Halgren, T.A., Klicic, J.J., Mainz, D.T., Repasky, M.P., Knoll, E.H., Shelley, M., Perry, J.K., Shaw, D.E., Francis, P., Shenkin, P.S., 2004. Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. J. Med. Chem. 47, 1739–1749. https://doi.org/10.1021/jm0306430.

Guzman-Gutierrez, S.L., Gomez-Cansino, R., Garcia-Zebadua, J.C., Jimenez-Perez, N.C., Reyes-Chilpa, R., 2012. Antidepressant activity of Litsea glaucescens essential oil: identification of beta-pinene and linalool as active principles. J. Ethnopharmacol. 143, 673–679. https://doi.org/10.1016/j.jep.2012.07.026.

Hadingham, K.L., Garrett, E.M., Wafford, K.A., Bain, C., Heavens, R.P., Sirinathsinghji, D.J., Whiting, P.J., 1996. Cloning of cDNAs encoding the human gamma-aminobutyric acid type A receptor alpha 6 subunit and characterization of the pharmacology of alpha 6-containing receptors. Mol. Pharmacol. 49, 253–259. https://www.ncbi.nlm.nih.gov/pubmed/8632757.

Hansel, R., Wohlfart, R., Schmidt, H., 1982. The sedativ-hypnotic principle of hops. Planta Med. 45, 224–228. https://doi.org/10.1055/s-2007-971377.

Hansel, R., Wohlfart, R., Coper, H., 1980. Sedative-hypnotic compounds in the exhalation of hops, II. Z. Naturforsch. C Biosci. 35, 1096–1097. https://www.ncbi.nlm.nih.gov/pubmed/7210807.

Hansel, R., Schulz, J., 1988. Desmethylxanthohumol: isolierung aus Hopfen und Cyclisierung zu Flavanonen. Arch. Pharm. Pharm. Med. Chem. 321, 37–40. https://doi.org/10.1002/ardp.19883210112.

Hossain, S.J., Hamamoto, K., Aoshima, H., Hara, Y., 2002. Effects of tea components on the response of GABA(A) receptors expressed in Xenopus Oocytes. J. Agric. Food Chem. 50, 3954–3960. https://doi.org/10.1021/jf011607h.

Huang, X., Liu, T., Gu, J., Luo, X., Ji, R., Cao, Y., Xue, H., Wong, J.T., Wong, B.L., Pei, G., Jiang, H., Chen, K., 2001. 3D-QSAR model of flavonoids binding at benzodiazepine site in GABAA receptors. J. Med. Chem. 44, 1883–1891. https://doi.org/10.1021/jm000557p.

Intelmann, D., Haseleu, G., Hofmann, T., 2009. LC-MS/MS Quantitation of hop-derived bitter compounds in beer using the ECHO technique. J. Agric. Food Chem. 57, 1172–1182. https://doi.org/10.1021/jf803040g.

Jackson, R.E.S., 1871. Notebook of Materia Medica, Pharmacology and Therapeutics. Maclachlan & Stewart, Edinburgh, pp. 521–522. https://books.google.com.mt/books?id=W9i-4wfm5poC.

Jechlinger, M., Pelz, R., Tretter, V., Klausberger, T., Sieghart, W., 1998. Subunit composition and quantitative Importance of hetero-oligomeric receptors: GABAA receptors containing α6 subunits. J. Neurosci. 18, 2449–2457. https://doi.org/10.1523/JNEUROSCI.18-07-02449.1998.

Jursky, F., Fuchs, K., Buhr, A., Tretter, V., Sigel, E., Sieghart, W., 2000. Identification of amino acid residues of GABA(A) receptor subunits contributing to the formation and affinity of the tert-butylbicyclophosphorothionate binding site. J. Neurochem. 74, 1310–1316. https://doi.org/10.1046/j.1471-4159.2000.741310.x.

Kaijima, M., Le Gal La Salle, G., Rossier, J., 1983. The partial benzodiazepine agonist properties of Ro 15-1788 in pentylenetetrazol-induced seizures in cats. Eur. J. Pharmacol. 93, 113–115. https://doi.org/10.1016/0014-2999(83)90037-7.

Karabín, M., Hudcová, T., Jelínek, L., Dostálek, P., 2016. Biologically active compounds from hops and prospects for their use. Compr. Rev. Food. Sci. F. 15, 542–567. https://doi.org/10.1111/1541-4337.12201.

Linck, V.M., da Silva, A.L., Figueiro, M., Caramao, E.B., Moreno, P.R., Elisabetsky, E., 2010. Effects of inhaled Linalool in anxiety, social interaction and aggressive behavior in mice. Phytomedicine 17, 679–683. https://doi.org/10.1016/j.phymed.2009.10.002.

Lyne, P.D., Lamb, M.L., Saeh, J.C., 2006. Accurate prediction of the relative potencies of members of a series of kinase inhibitors using molecular docking and MM-GBSA scoring. J. Med. Chem. 49, 4805–4808. https://doi.org/10.1021/jm060522a.

Maldifassi, M.C., Baur, R., Sigel, E., 2016. Molecular mode of action of CGS 9895 at alpha1 beta2 gamma2 $GABA_A$ receptors. J. Neurochem. 138, 722–730. https://doi.org/10.1111/jnc.13711.

Meissner, O., Häberlein, H., 2006. Influence of Xanthohumol on the binding behavior of $GABA_A$ receptors and their lateral mobility at hippocampal neurons. Planta Med. 72, 656–658. https://doi.org/10.1055/s-2006-931609.

Milanos, S., Elsharif, S.A., Janzen, D., Buettner, A., Villmann, C., 2017. Metabolic products of linalool and modulation of GABAA receptors. Front. Chem. 5, 46. https://doi.org/10.3389%2Ffchem.2017.00046.

Miles, C.O., Main, L., 1985. Kinetics and mechanism of the cyclisation of 2′,6′-dihydroxy-4,4′-dimethoxy-chalcone; influence of the 6′-hydroxy group on the rate of cyclisation under neutral conditions. J. Chem. Soc., Perkin Trans. 2, 1639–1642. https://doi.org/10.1039/P29850001639.

Milligan, S.R., Kalita, J.C., Heyerick, A., Rong, H., De Cooman, L., De Keukeleire, D., 1999. Identification of a potent phytoestrogen in hops (Humulus lupulus L.) and beer. J. Clin. Endocrinol. Metab. 84, 2249–2252. https://doi.org/10.1210/jcem.84.6.5887.

Mizobuchi, S., Sato, Y., 1984. A new flavanone with antifungal activity isolated from hops. Agric. Biol. Chem. 48, 2771–2775. https://doi.org/10.1080/00021369.1984.10866564.

Peacock, V.E., Deinzer, M.L., 1981. Chemistry of hop aroma in beer. J. Am. Soc. Brew. Chem. 39, 136–141. https://doi.org/10.1094/ASBCJ-39-0136.

Prencipe, F.P., Brighenti, V., Rodolfi, M., Mongelli, A., dall'Asta, C., Ganino, T., Bruni, R., Pellati, F., 2014. Development of a new high-performance liquid chromatography method with diode array and electrospray ionization-mass spectrometry detection for the metabolite fingerprinting of bioactive compounds in Humulus lupulus L. J. Chromatogr. A 1349, 50–59. https://doi.org/10.1016/j.chroma.2014.04.097.

Pirker, S., Schwarzer, C., Wieselthaler, A., Sieghart, W., Sperk, G., 2000. $GABA_A$ receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101, 815–850. https://doi.org/10.1016/s0306-4522(00)00442-5.

Pöltl, A., Hauer, B., Fuchs, K., Tretter, V., Sieghart, W., 2003. Subunit composition and quantitative importance of $GABA_A$ receptor subtypes in the cerebellum of mouse and rat. J. Neurochem. 87, 1444–1455. https://doi.org/10.1046/j.1471-4159.2003.02135.x.

Quirk, K., Whiting, P.J., Ragan, C.I., McKernan, R.M., 1995. Characterisation of delta-subunit containing $GABA_A$ receptors from rat brain. Eur. J. Pharmacol. 290, 175–181. https://doi.org/10.1016/0922-4106(95)00061-5.

Ramerstorfer, J., Furtmuller, R., Sarto-Jackson, I., Varagic, Z., Sieghart, W., Ernst, M., 2011. The $GABA_A$ receptor alpha + beta- interface: a novel target for subtype selective drugs. J. Neurosci. 31, 870–877. https://doi.org/10.1523/JNEUROSCI.5012-10.2011.

Renard, S., Olivier, A., Granger, P., Avenet, P., Graham, D., Sevrin, M., George, P., Besnard, F., 1999. Structural elements of the gamma-aminobutyric acid type A receptor conferring subtype selectivity for benzodiazepine site ligands. J. Biol. Chem. 274, 13370–13374. https://doi.org/10.1074/jbc.274.19.13370.

Richter, L., de Graaf, C., Sieghart, W., Varagic, Z., Morzinger, M., de Esch, I.J., Ecker, G.F., Ernst, M., 2012. Diazepam-bound $GABA_A$ receptor models identify new benzodiazepine binding-site ligands. Nat. Chem. Biol. 8, 455–464. https://doi.org/10.1038%2Fnchembio.917.

Sahin, S., Eulenburg, V., Kreis, W., Villmann, C., Pischetsrieder, M., 2016. Three-step test system for the identification of novel $GABA_A$ receptor modulating food plants. Plant Foods Hum. Nutr. 71, 355–360. https://doi.org/10.1007/s11130-016-0566-1.

Sastry, G.M., Inakollu, V.S., Sherman, W., 2013. Boosting virtual screening enrichments with data fusion: coalescing hits from two-dimensional fingerprints, shape, and docking. J. Chem. Inf. Model. 53, 1531–1542. https://doi.org/10.1021/ci300463g.

Schiller, H., Forster, A., Vonhoff, C., Hegger, M., Biller, A., Winterhoff, H., 2006. Sedating effects of Humulus lupulus L. extracts. Phytomedicine 13, 535–541. https://doi.org/10.1016/j.phymed.2006.05.010.

Sekiguchi, F., Fujita, T., Deguchi, T., Yamaoka, S., Tomochika, K., Tsubota, M., Ono, S., Horaguchi, Y., Ichii, M., Ichikawa, M., Ueno, Y., Koike, N., Tanino, T., Nguyen, H.D., Okada, T., Nishikawa, H., Yoshida, S., Ohkubo, T., Toyooka, N., Murata, K., Matsuda, H., Kawabata, A., 2018. Blockade of T-type calcium channels by 6-prenylnaringenin, a hop component, alleviates neuropathic and visceral pain in mice. Neuropharmacology 138, 232–244. https://doi.org/10.1016/j.neuropharm.2018.06.020.

Sieghart, W., 1995. Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. Pharmacol. Rev. 47, 181–234. https://www.ncbi.nlm.nih.gov/pubmed/7568326.

Sherman, W., Beard, H.S., Farid, R., 2006a. Use of an induced fit receptor structure in virtual screening. Chem. Biol. Drug Des. 67, 83–84. https://doi.org/10.1111/j.1747-0285.2005.00327.x.

Sherman, W., Day, T., Jacobson, M.P., Friesner, R.A., Farid, R., 2006b. Novel procedure for modeling ligand/receptor induced fit effects. J. Med. Chem. 49, 534–553. https://doi.org/10.1021/jm050540c.

Smith, R.J., Wilson, R.J., 1992. Hops-derived foam stabilizing and bittering agents. WO1993002177A1. https://patents.google.com/patent/WO1993002177A1.

Squires, R.F., Saederup, E., 2000. Additivities of compounds that increase the numbers of high affinity [^{3}H]muscimol binding sites by different amounts define more than 9 GABA(A) receptor complexes in rat forebrain: implications for schizophrenia and clozapine research. Neurochem. Res. 25, 1587–1601. https://doi.org/10.1023/A%3A1026666419725.

Štěrba, K., Čejka, P., Čulík, J., Jurková, M., Krofta, K., Pavlovič, M., Mikyška, A., Olšovská, J., 2015. Determination of linalool in different hop varieties using a new method based on fluidized-bed extraction with gas chromatographic-mass spectrometric detection. J. Am. Soc. Brew. Chem. 73, 151–158. https://doi.org/10.1094/ASBCJ-2015-0406-01.

Stevens, J.F., Taylor, A.W., Clawson, J.E., Deinzer, M.L., 1999. Fate of xanthohumol and related prenylflavonoids from hops to beer. J. Agric. Food Chem. 47, 2421–2428. https://doi.org/10.1021/jf990101k.

Uusi-Oukari, M., Korpi, E.R., 1992. Functional properties of $GABA_A$ receptors in two rat lines selected for high and low alcohol sensitivity. Alcohol 9, 261–269. https://doi.org/10.1016/0741-8329(92)90063-G.

Van Cleemput, M., Cattoor, K., De Bosscher, K., Haegeman, G., De Keukeleire, D., Heyerick, A., 2009. Hop (Humulus lupulus)-derived bitter acids as multipotent bioactive compounds. J. Nat. Prod. 72, 1220–1230. https://doi.org/10.1021/np800740m.

Varagic, Z., Wimmer, L., Schnurch, M., Mihovilovic, M.D., Huang, S., Rallapalli, S., Cook, J.M., Mirheydari, P., Ecker, G.F., Sieghart, W., Ernst, M., 2013. Identification of novel positive allosteric modulators and null modulators at the $GABA_A$ receptor alpha + beta- interface. Br. J. Pharmacol. 169, 371–383. https://doi.org/10.1111%2Fbph.12151.

Vellucci, S.V., Webster, R.A., 1983. Is Ro15-1788 a partial agonist at benzodiazepine receptors? Eur. J. Pharmacol. 90, 263–268. https://doi.org/10.1016/0014-2999(83)90247-9.

Venturelli, S., Niessner, H., Sinnberg, T., Berger, A., Burkard, M., Urmann, C., Donaubauer, K., Bocker, A., Leischner, C., Riepl, H., Frank, J., Lauer, U.M., Garbe, C., Busch, C., 2018. 6- and 8-prenylnaringenin, novel natural histone deacetylase inhibitors found in hops, exert antitumor activity on melanoma cells. Cell. Physiol. Biochem. 51, 543–556. https://doi.org/10.1159/000495275.

Verzele, M., Anteunis, M., Alderweireldt, F., 1965. Isohumulones*. J. Inst. Brew. 71, 232–239. https://doi.org/10.1002/j.2050-0416.1965.tb02051.x.

Walters, W.P., Stahl, M.T., Murcko, M.A., 1998. Virtual screening—an overview. Drug Discov. Today 3, 160–178. https://doi.org/10.1016/S1359-6446(97)01163-X.

Wohlfart, R., Hansel, R., Schmidt, H., 1983a. The sedative-hypnotic action of hops. 4. Pharmacology of the hop substance 2-methyl-3-buten-2-ol. Planta Med. 48, 120–123. https://www.ncbi.nlm.nih.gov/pubmed/17396916.

Wohlfart, R., Wurm, G., Hansel, R., Schmidt, H., 1983b. Detection of sedative-hypnotic active ingredients in hops. 5. Degradation of bitter acids to 2-methyl-3-buten-2-ol, a hop constituent with sedative-hypnotic activity. Arch. Pharm. (Weinheim) 316, 132–137. https://www.ncbi.nlm.nih.gov/pubmed/6847343.

Zanoli, P., Zavatti, M., Rivasi, M., Brusiani, F., Losi, G., Puia, G., Avallone, R., Baraldi, M.,

2007. Evidence that the beta-acids fraction of hops reduces central GABAergic neurotransmission. J. Ethnopharmacol. 109, 87–92. https://doi.org/10.1016/j.jep.2006.07.008.

Zanoli, P., Rivasi, M., Zavatti, M., Brusiani, F., Baraldi, M., 2005. New insight in the neuropharmacological activity of Humulus lupulus L. J. Ethnopharmacol. 102, 102–106. https://doi.org/10.1016/j.jep.2005.05.040.

Zhang, X., Liang, X., Xiao, H., Xu, Q., 2004. Direct characterization of bitter acids in a crude hop extract by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J. Am. Soc. Mass Spectrom. 15, 180–187. https://doi.org/10.1016/j.jasms.2003.09.014.

Zhu, S., Noviello, C.M., Teng, J., Walsh Jr., R.M., Kim, J.J., Hibbs, R.E., 2018. Structure of a human synaptic $GABA_A$ receptor. Nature 559, 67–72. https://doi.org/10.1038/s41586-018-0255-3.