A cycloartane glycoside derived from Actaea racemosa L. modulates GABA_A receptors and induces pronounced sedation in mice

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Ac-SM (23-O-Acetylshengmanol 3-O-β-D-xylopyranoside)
Abstract

23-O-acetylshengmanol-3-O-β-d-xylopyranoside (Ac-SM) isolated from Actaea racemosa L. - an herbal remedy for the treatment of mild menopausal disorders- has been recently identified as a novel efficacious modulator of γ-aminobutyric acid (GABA) type A (GABAA) receptors composed of α1-, β2- and γ2S-subunits. In the present study, we analyzed a potential subunit-selective modulation of GABA-induced chloride currents (IGABA EC3-8) through nine GABAA receptor isoforms expressed in Xenopus laevis oocytes by Ac-SM with 2-microelectrode-voltage-clamp and behavioral effects 30 min after intraperitoneal application in a mouse model. Efficacy of IGABA enhancement by Ac-SM displayed a mild α-subunit-dependence with α2β2γ2S (maximal IGABA potentiation E_max=1454±97%) and α5β2γ2S (E_max=1408±87%) receptors being most efficaciously modulated, followed by slightly weaker IGABA enhancement through α1β2γ2S (E_max=1187±166%), α3β2γ2S (E_max=1174±218%) and α6β2γ2S (E_max=1171±274%) receptors and less pronounced effects on receptors composed of α4β2γ2S (E_max=752±53%) subunits, while potency was not affected by the subunit composition (EC50 values ranging from α1β2γ2S=35.4±12.3µM to α5β2γ2S=50.9±11.8µM). Replacing β2- by β1- or β3-subunits as well as omitting the γ2S-subunit affected neither efficacy nor potency of IGABA enhancement by Ac-SM. Ac-SM shifted the GABA-concentration-response-curve towards higher GABA sensitivity (about 3-fold) and significantly increased the maximal GABA response by 44±13% indicating pharmacological profile distinct from a pure allosteric GABAA receptors modulator. In mice, Ac-SM significantly reduced anxiety-related behavior in the elevated-plus-maze-test at a dose of 0.6mg/kg, total ambulation in the open-field-test at doses ≥6mg/kg, stress-induced hyperthermia at doses ≥0.6mg/kg,
and significantly elevated seizure threshold at doses ≥20mg/kg bodyweight. High efficacy and long biological half-life of Ac-SM suggest that potential cumulative sedative side effects upon repetitive intake of *Actaea racemosa* L. preparations might not be negligible.

**Introduction**
γ-aminobutyric acid (GABA) mediates fast inhibitory neurotransmission in the mammalian brain by activating synaptic and extrasynaptic GABA type A (GABA<sub>A</sub>) receptors. GABA<sub>A</sub> receptors are composed of five subunits that form a chloride conducting ion pore (Macdonald and Olsen, 1994; Sieghart, 1995; Sieghart and Sperk, 2002; Sigel and Steinmann, 2012). 19 different GABA<sub>A</sub> receptor subunits have been identified in the human genome (Simon et al., 2004) allowing theoretically the formation of approximately 800 subunit combinations. However, so far the existence of only 11 native GABA<sub>A</sub> receptors subtypes has been confirmed (Olsen and Sieghart, 2008). There is consensus that the mainly expressed GABA<sub>A</sub> receptor isoform consists of α<sub>1</sub>, β<sub>2</sub>, and γ<sub>2</sub> subunits (Olsen and Sieghart, 2008) with a commonly accepted 2α:2β:1γ subunit stoichiometry (Tretter et al., 1997; Baumann et al., 2001, 2002). The subunit combination determines the pharmacological properties of GABA<sub>A</sub> receptors (Olsen and Sieghart, 2009). GABA<sub>A</sub> receptors play a major role in controlling the excitability of the mammalian brain and are thus involved in regulating amongst others sleep, vigilance, mood and emotions (Möhler et al., 2005; Möhler, 2006). GABA<sub>A</sub> receptors are modulated by structurally diverse compounds including clinically applied drugs such as benzodiazepines, barbiturates, neurosteroids, anaesthetics as well as a large number of natural products including flavonoids, terpenoids or polyacetlyenes (for review see Johnston et al., 2006; Olsen and Sieghart, 2009; Hanrahan et al., 2011; Nilsson and Sterner, 2011). Recently, we have identified four cycloartane glycosides (actein, cimigenol 3-O-β-D-xylopyranoside, 25-O-acetylcimigenol 3-O-α-L-arabinopyranoside and 23-O-acetylshengmanol 3-O-β-D-xylopyranoside) as novel efficacious GABA<sub>A</sub> modulators isolated from Actaea racemosa L. While I<sub>GABA</sub> enhancement by actein,
cimigenol 3-\(\text{O-}\beta-\text{d-xylopyranoside}\) and 25-\(\text{O-}\text{acetyl}\text{cimigenol 3-}\text{O-}\alpha-\text{l-arabinopyranoside}\) averaged at about 300\%, a more than 5-fold stronger \(I_{\text{GABA}}\) potentiation was observed for the cycloartane glycoside 23-\(\text{O-}\text{acetylshengmanol 3-}\text{O-}\beta-\text{d-xylopyranoside (Ac-SM)}\) (Cicek et al., 2010).

The aim of the present study was to obtain deeper insights into the molecular mechanism of Ac-SM action by analyzing a potential subunit-dependent modulation of nine different \(\text{GABA}_A\) receptor subtypes expressed in \textit{Xenopus laevis} oocytes. In addition, potential anxiolytic, stress-reducing, anticonvulsant effects as well as changes in locomotor activity upon application of Ac-SM were studied in mice to investigate the potential effects of this compound on mood, sedation and seizure threshold.
Materials and Methods

Animals

All procedures involving animals were approved by the Austrian Animal Experimentation Ethics Board in compliance with the European convention (Directive 2010/63/EU) for the protection of vertebrate animals used for experimental and other scientific purposes ETS no.: 123. Every effort was taken to minimize the number of animals used.

Chemicals

Ac-SM (Fig. 1) was isolated from Actaea racemosa L. as previously described (Cicek et al., 2010). Stock solutions (100mM for in vitro experiments and 1mg/10µl for in vivo experiments, respectively) were prepared in dimethylsulfoxide (DMSO). All chemicals were obtained from Sigma Aldrich (Vienna, Austria).

Due to low solubility in the bath solution Ac-SM was used only up to a concentration of 300µM in in vitro experiments. Equal amounts of DMSO were present in control and compound-containing solutions. The maximum DMSO concentration in the bath (0.3%) did not affect IgABA (Khom et al., 2006).

For in vivo experiments, working concentrations were adjusted by dilution with 0.9% sodium chloride; the final concentration of DMSO was fixed to 10% including control solutions. To enhance solubility of the compound Tween 80 (3% final concentration) was added to all solutions. pH was adjusted to 7.2-7.4 with 1M sodium hydroxide. All solutions were freshly prepared every day prior to experiments.
Expression and functional characterization of GABA_A receptors

Preparation of stage V-VI oocytes from *Xenopus laevis* (NASCO, Fort Atkinson, USA), synthesis of capped off run-off poly(A^+) rat cRNA transcripts from linearized cDNA templates (pCMV vector) was performed as described (Khom *et al.*, 2006). Briefly, female *Xenopus laevis* were anaesthetized by exposing them for 15 min to a 0.2% solution of MS-222 (methane sulfonate salt of 3-aminobenzoic acid ethyl ester) before surgically removing parts of the ovaries. Follicle membranes from isolated oocytes were enzymatically digested with 2 mg/ml collagenase (Type 1A). Oocytes were stored at 18°C in ND96 solution (Methfessel *et al.*, 1986). One day after isolation, the oocytes were injected with about 10-50 nl of diethyl pyrocarbonate (DEPC) - treated water containing the different rat cRNAs (150 - 3000 ng/μl /subunit). To ensure expression of the γ_2S-subunit in the case of α_1β_2γ_2S, α_2β_2γ_2S, α_3β_2γ_2S, α_6β_2γ_2S and α_1β_3γ_2S receptors, cRNAs were mixed in a ratio of 1:1:10 (Boileau *et al.*, 2002) except for α_4β_2γ_2S receptors a ratio 3:1:5 was used. For α_1β_2 receptors, cRNAs were mixed in a ratio 1:1. cRNAs for α_1β_1γ_2S channels were injected in a ratio of 3:1:10 to avoid formation of β_1-homooligomeric GABA_A receptors (Krishek *et al.*, 1996). The amount of cRNA was determined by means of a NanoDrop ND-1000 (Kisker-biotech, Steinfurt, Germany).

Electrophysiological experiments were done using the two-microelectrode-voltage-clamp-technique at a holding potential of -70 mV making use of a TURBO TEC 01C amplifier (npi electronic, Tamm, Germany) and an Axon Digidata 1322A interface (Molecular Devices, Sunnyvale, CA). Data acquisition was carried out using pCLAMP v.9.2 (Molecular Devices, Sunnyvale, CA). The bath solution contained 90 mM NaCl,
1 mM KCl, 1 mM MgCl₂·6H₂O, 1 mM CaCl₂ and 5 mM HEPES (pH 7.4). Microelectrodes were filled with 2 M KCl and had resistances between 1 and 3 MΩ (Khom et al., 2006).

**Perfusion system**

GABA and Ac-SM were applied by means of fast perfusion system (for details see Baburin et al., 2006). Drug or control solutions were applied by means of a TECAN Miniprep 60 permitting automation of the experiments. To elicit I₉GABA the chamber was perfused with 120 µl of GABA-containing solution at volume rate between 300 and 1000 µl/s. The I₉GABA rise time ranged between 100 and 250 ms (Khom et al., 2006). To account for possible slow recovery from increasing levels of desensitization in the presence of high GABA or compound concentrations, the duration of washout periods was extended stepwise, i.e. 1.5 min (GABA EC₃₋₈) to 3 min (co-application of GABA EC₃₋₈ in the presence ≤ 1 µM Ac-SM;) to 5-10 min (co-application of GABA EC₃₋₈ in the presence of ≤ 10 µM compound) to 15-20 min (co-application of GABA EC₃₋₈ and ≤ 100 µM Ac-SM) to 30 min (GABA EC₃₋₈ in the presence of 300 µM Ac-SM). The duration of washout periods in experiments analyzing the effect of Ac-SM (300 µM) at different GABA concentrations was fixed to 30 min. Potential run-down or run-up effects were ruled out by application of GABA control at the end of each experiment. Oocytes with maximal current amplitudes > 3 µA were discarded to exclude voltage-clamp errors (Khom et al., 2006).
**Analyzing concentration-response curves**

Stimulation of chloride currents by modulators of the GABA<sub>A</sub> receptor was measured at a GABA concentration eliciting between 3 and 8% of the maximal current amplitude (EC<sub>3-8</sub>). The EC<sub>3-8</sub> was determined at the beginning of the experiment for each oocyte by application of 1mM GABA followed by submaximal GABA concentrations.

Enhancement of the chloride current was defined as \( \frac{I_{(GABA+Comp)}}{I_{GABA}} - 1 \), where \( I_{(GABA+Comp)} \) is the current response in the presence of Ac-SM and \( I_{GABA} \) is the control GABA current. To measure the sensitivity of the GABA<sub>A</sub> receptor for a given compound, it was applied for an equilibration period of 1min before applying GABA (EC<sub>3-8</sub>). Concentration-response curves were generated and the data were fitted by non-linear regression analysis using Origin software (OriginLab Corporation, USA). Data were fitted to the equation:

\[
\frac{1}{1 + \left( \frac{EC_{50}}{(Comp)} \right)^{n_H}},
\]

where \( n_H \) is the Hill coefficient. Each data point represents the mean±SEM from at least 3 oocytes and \( \geq 2 \) oocyte batches.

**Animals**

Male mice (C57BL/6N) were obtained from Charles River Laboratories (Sulzfeld, Germany). For maintenance, mice were group-housed (maximum 5 mice per type IIL cage) with free access to food and water. At least 24h before the commencement of experiments, mice were transferred to the testing facility, continuing *ad libitum* access to food and water. The temperature in the holding and testing facilities was 22±2°C; the humidity was 40-60%; a 12h light-dark cycle was in operation (lights on from 07.00 to 19.00). Male mice aged 3-6 months were tested.
Intraperitoneal (i.p.) injection of control or Ac-SM-containing solutions was usually done 30 min before the test, except for home cage (HC) activity analysis (60 min before test) and measurement of stress-induced hyperthermia (3 h before test) to reduce the impact of stress on the analysis. Application of the solvent alone did not influence animal behavior as compared to saline-treated controls. All doses are indicated as mg/kg bodyweight of the animal.

**Open-field-test**

Exploration of a novel environment was tested over 10 min in a 50x50 cm box build from gray PVC equipped with infra-red beams. Illumination intensity was set to 150 lux in the center. Animals' explorative behavior was analyzed using ActiMot-2 equipment and software (TSE-systems, Bad Homburg, Germany). Arenas were subdivided into 3 fields: border (up to 8 cm from wall), center (20x20 cm, i.e. 16% of total area), and intermediate area according to the recommendations of EMPRESS (European Mouse Phenotyping Resource of Standardised Screens; http://empress.har.mrc.ac.uk).

**Home Cage Activity (HC)**

For HC activity mice were singly placed into a type IIIL cage with free access to food and water, enriched with a plastic tube. Movement of mice was monitored through an infra-red detection system (InfraMot, TSE-systems, Bad Homburg, Germany). HC behavior analysis was started at 5 pm and ran for 72 h. Arbitrary activity counts (AAC) were cumulated in 60 minutes intervals.
**Elevated-plus-maze (EPM)-test**

The animal’s behavior was tested over 5 min on an elevated plus maze 1 m above ground. The EPM consisted of 2 closed arms (walls 20 cm in height) and 2 open arms, facing each other. The test instrument was built from gray PVC, each arm 50x5 cm in size. Illumination intensity was set to 180 lux at the intersection of the arms (defined as the neutral field). Animals were placed into the center, facing an open arm (OA). Analysis of open arm entries time on open arms and distance on the open arms was automatically done with Video-Mot 2 equipment and software (Wittmann *et al.*, 2009).

**Stress-induced-hyperthermia-test**

Stress-induced hyperthermia (SIH) was assessed as described previously (Wittmann *et al.*, 2009). In short: a temperature probe, lubricated with glycerol, was inserted into the rectum of the mouse to a depth of up to 2 cm to measure basal temperature T1. The temperature probe remained in the animal till a stable temperature was reached (maximum 10 s). Measurement of T1 served also as the handling stressor. After 15 min the measurement was repeated (T2) and the rise in temperature (delta T) was considered as stress-induced hyperthermia (Olivier *et al.*, 2003).

**Seizure threshold**

Seizure threshold was determined by pentylenetetrazole (PTZ) tail-vein infusion on freely moving animals at a rate of 100 µl/min (10 mg/ml PTZ in saline, pH = 7.4). Infusion was stopped when animals displayed generalized clonic seizures. Animals were immediately
killed by cervical displacement after onset of seizures. The seizure threshold dose was calculated from the infused volume in relation to body weight.

**Statistical analysis**

Statistical significance was calculated by one-way ANOVA and where appropriate by two-way ANOVA (followed by a post-hoc mean comparison with Bonferroni; GraphPad; La Jolla, CA; USA). *P*-values of <0.05 were accepted as statistically significant. All data are given as mean±SEM.
Results

In the present study we demonstrate a comparable potency and efficacy of $I_{\text{GABA}}$ modulation by Ac-SM (for structural formulae, see Fig.1) on nine different GABA<sub>A</sub> receptor subtypes. $I_{\text{GABA}}$ modulation was independent of the presence of a $\gamma_{2S}$-subunit. Ac-SM induces anxiolytic, stress-reducing, sedative and anticonvulsant effects.

**$I_{\text{GABA}}$ enhancement by Ac-SM does not require the presence of a $\gamma_{2S}$-subunit**

Ac-SM efficaciously potentiates $I_{\text{GABA}}$ through GABA<sub>A</sub> receptors composed of $\alpha_1\beta_2\gamma_{2S}$ subunits (Cicek et al., 2010). As illustrated in Fig.2A, Ac-SM, however, also modulated GABA<sub>A</sub> receptors lacking a $\gamma_{2S}$-subunit with similar efficacy and potency ($E_{\text{max}}(\alpha_1\beta_2\gamma_{2S})$: 1187±166% and $EC_{50}(\alpha_1\beta_2\gamma_{2S})$: 47.1±20.9µM vs. $E_{\text{max}}(\alpha_1\beta_2)$: 1277±243% and $EC_{50}(\alpha_1\beta_2\gamma_{2S})$: 45.0±11.4µM; see also Table 1).

**Ac-SM enhances $I_{\text{GABA}}$ irrespective of the GABA<sub>A</sub> subunit composition**

In order to investigate a possible $\alpha$-subunit-dependent $I_{\text{GABA}}$ enhancement, the $\alpha_1$-subunit was substituted by $\alpha_2$-, $\alpha_3$-, $\alpha_4$-, $\alpha_5$- and $\alpha_6$-, respectively and expressed in combination with $\beta_2$- and $\gamma_{2S}$-subunits (Fig.2B, Table 1). The highest efficacy was observed for receptors containing either $\alpha_2$- or $\alpha_5$-subunits, with a maximal $I_{\text{GABA}}$ potentiation of $E_{\text{max}}(\alpha_2\beta_2\gamma_{2S})$: 1454±97% (n=7) and $E_{\text{max}}(\alpha_5\beta_2\gamma_{2S})$: 1408±87% (n=3), respectively (Table 1), followed by receptors comprising either $\alpha_1$- ($E_{\text{max}}(\alpha_1\beta_2\gamma_{2S})$: 1187±166%; n=6), $\alpha_3$- ($E_{\text{max}}(\alpha_3\beta_2\gamma_{2S})$: 1174±218%; n=4) or $\alpha_6$-subunits ($E_{\text{max}}(\alpha_6\beta_2\gamma_{2S})$: 1171±274%; n=3). Ac-SM, however, displayed a lower efficacy on GABA<sub>A</sub> receptors comprising $\alpha_4$-subunits.
compared to $\alpha_1\beta_2\gamma_2S$, $\alpha_3\beta_2\gamma_2S$, $\alpha_6\beta_2\gamma_2S$ ($p<0.05$), $\alpha_2\beta_2\gamma_2S$ and $\alpha_5\beta_2\gamma_2S$ ($p<0.001$) receptors ($E_{\text{max}}(\alpha_4\beta_2\gamma_2S)752\pm53\%$; n=3; $F_{5,20}=9.185$; Table1).

In contrast to this moderate $\alpha$-subunit-dependence, replacing the $\beta_2$-subunit in $\alpha_1\beta_2\gamma_2S$ receptors by either $\beta_1$- or $\beta_3$-subunits (Fig.2C), did not affect the strength of $I_{\text{GABA}}$ enhancement by Ac-SM ($E_{\text{max}}(\alpha_1\beta_2\gamma_2S): 1069\pm214\%$, n=6 vs. $E_{\text{max}}(\alpha_1\beta_2\gamma_2S): 1187\pm166\%$, n=6 vs. $E_{\text{max}}(\alpha_1\beta_3\gamma_2S): 1247\pm222\%$, n=4).

Comparison of the potencies of the respective GABA$_A$ receptor isoforms revealed no significant differences, with EC$_{50}$ values ranging from $35.4\pm12.3\mu M$ ($\alpha_1\beta_3\gamma_2S$) to $50.9\pm11.8\mu M$ ($\alpha_5\beta_2\gamma_2S$) (see Table 1). Typical current traces for concentration-dependent $I_{\text{GABA}}$ enhancement by Ac-SM are illustrated in Fig.2D.

**Ac-SM shifts the GABA-concentration-response curves and enhances $I_{\text{GABA}}$-max**

To gain further insight into the mechanism of $I_{\text{GABA}}$ modulation by Ac-SM, we compared GABA-concentration-response curves in the presence and absence of Ac-SM on $\alpha_1\beta_3$ channels. Ac-SM was applied at a saturating concentration of 300µM. At this concentration, Ac-SM shifted the concentration-effect curve towards significantly lower GABA concentrations ($EC_{50(\text{GABA})}: 3.7\pm0.4\mu M$ vs. $EC_{50(\text{GABA}+\text{Ac-SM})}: 1.2\pm0.2\mu M$, $p<0.01$) and also increased the maximal GABA response by 44\% (n=3, see Fig.3). Ac-SM at 300µM induced only small currents in the absence of GABA (not exceeding 1\% of maximal $I_{\text{GABA}}$ induced by 1mM GABA) indicating modulatory effects (see also (Cicek et al., 2010)).

**Ac-SM dose-dependently reduces locomotor activity**
In order to get first insight into the in vivo effects of Ac-SM, the explorative behavior of male C57BL/6N mice was studied 30min after intraperitoneal application in the open-field-test (OF). As illustrated in Fig.4A, no changes in total ambulation were observed upon application of Ac-SM at doses of ≤2mg/kg. Reduced ambulation compared to solvent-treated controls started at doses ≥6mg/kg reaching a maximum at a dose of 60mg/kg by reducing the total distance by more than 60% (Control: 38.0±1.2m vs. Ac-SM 60mg/kg: 14.7±3.1m; \( F_{(6,84)}=19.597, p<0.01 \)). Time spent in the center of the OF did not significantly differ between compound-treated (doses ≤20mg/kg) and control group. Only mice treated with Ac-SM at a dose of 60mg/kg spent significantly more time in the center area of the OF (Control: 57.7±6.1s vs. Ac-SM 60mg/kg: 212.5±101.6s; \( F_{(6,84)}=2.815, p<0.05 \); see Fig.4B) reflecting primarily the strong suppression of motor activity. The number of entries into the center area mostly paralleled the time spent in this compartment. However, mice treated with 0.2mg/kg Ac-SM tended to visit the center area more frequently compared to the control group (Control: 28.2±2.1 vs. Ac-SM 0.6mg/kg: 33.1±4.1). In line with reduced ambulation also the number of center entries significantly dropped at a dose of 60mg/kg (Control: 28.2±2.1 vs. Ac-SM 60mg/kg: 5.9±1.6; \( F_{(6,84)}=8.828, p<0.01 \); see Fig.4C). Control mice and Ac-SM-treated mice covered a comparable percentage of total distances in the center area (see Fig.4D). Higher doses than 60mg/kg were not tested due to limited solubility of the compound.

To investigate the duration of in vivo Ac-SM effects, we investigated locomotor activity of mice treated with a dose inducing marked reduction of ambulation. Home cage activity analysis (Fig. 5) revealed a long lasting reduction of motor activity at a dose of 20mg/kg for at least 6-12h. No rebound effect on the subsequent time periods was measured (2-
way ANOVA, drug effect: $F_{(1,670)}=16.81, \ p<0.0001$; time effect: $F_{(66,670)}=5.77, \ p<0.0001$).

**Ac-SM paradigm-dependently reduces anxiety-related behavior and stress-induced hyperthermia**

Ac-SM slightly reduced motor activity at doses >2mg/kg in the OF compared to vehicle (see Fig.4A). Thus, to assess changes in anxiety-related behavior, the effect of lower doses was analyzed in the elevated-plus-maze-test: as illustrated in Fig.6A, Ac-SM-treated mice (0.6mg/kg) visited the open arms (OA) of the EPM more frequently (control: 15.0±1.6% of total entries vs. Ac-SM 0.6mg/kg: 28.3±4.5% of total entries; $F_{(2,37)}=4.667; \ p<0.05$). In addition, a tendency towards more time spent (Fig.6B) and longer distances covered on the OA compared to control animals was observed (Fig.6C), these effects were, however, not statistically significant. Total motor activity was not affected at these doses (data not shown).

Studying potential anxiolytic effects at higher doses was, however, hampered by the concomitantly occurring reduction of motor activity that might easily mask anxiolytic effects, as the behavioral tests (OF and EPM) rely on exploratory behavior. Therefore, we included a locomotion-independent test of a physiological stress response. Activation of the autonomic nervous system in response to stress such as exposure to noise, heat or pain in animals can be measured by changes in body temperature- a process referred to as stress-induced hyperthermia (SIH). SIH is a short lasting body temperature elevation ($\Delta T$) that can be reduced or even ablated by anxiolytic drugs (Vinkers et al., 2008). Basal
body temperature measured before compound application did not differ between control and compound-treated mice (data not shown).

As shown in Fig.7, body temperature upon stress exposure (i.e. insertion of the temperature probe) was raised in saline-treated control mice by 1.2±0.1°C. 3 hours after application, Ac-SM at doses ≥0.6mg/kg significantly reduced ΔT in a dose-dependent manner (0.6mg/kg: ΔT: 0.6±0.1°C; $F_{(4,41)}=15.350; p<0.01$). The maximal effect was reached at a dose of 6mg/kg ($ΔT=0.1±0.1°C; F_{(4,41)}=15.350; p<0.01$), where SIH was not traceable any more.

**Ac-SM increases the seizure threshold upon pentylenetetrazole infusion.**

A common feature of most positive allosteric modulators of GABA$_A$ receptors is an increased threshold against pentylenetetrazole-induced seizures (De Deyn and Macdonald, 1989; Rudolph *et al.*, 1999; Gerak and France, 2011; Löscher *et al.*, 2013). Indeed, Ac-SM significantly increased the seizure threshold at doses ≥20mg/kg (Control: 40.4±3.5mg/kg PTZ; vs. Ac-SM 20mg/kg: 58.5±1.7mg/kg PTZ; $F_{(6,23)}=19.903, p<0.001$; see Fig.8).
Discussion

Black cohosh (*Actaea racemosa* L.) preparations are widely used, mainly to relieve menopausal symptoms, although their efficacy is still controversially discussed (Borrelli and Ernst, 2008; Rees, 2009; Sassarini and Lumsden, 2010; Bedell et al., 2012, 2014; Wuttke et al., 2013). Despite extensive studies on the plants’ potential active principles, its mechanism of action is still not entirely understood (Palacio et al., 2009; Wuttke et al., 2013; Bedell et al., 2014). Understanding its molecular mechanism(s) of action is, however, a *conditio sine qua non* for considering potential beneficial and unwanted side effects. The initially suggested estrogenic activity of lipophilic components such as triterpenoids was disproved by later studies (Gaube et al., 2007; Mercado-Feliciano et al., 2012; Hajirahimkhan et al., 2013). There is, however, evidence that *Actaea racemosa* L. preparations interact with distinct neurotransmitter systems in the central nervous system including µ-opiod receptors, (Rhyu et al., 2006; Reame et al., 2008), serotonin (5-HT)-receptors (subtypes 5-HT1A, 5-HT1D and 5-HT7 (Burdette et al., 2003) and dopamine subtype 2 (D2) receptors (Jarry et al., 2003). We have previously shown that *Actaea racemosa* L. extracts efficaciously modulate GABA<sub>A</sub> receptors and identified four structurally related cycloartane glycosides in *Actaea racemosa* L. extracts as novel class of GABA<sub>A</sub> receptor ligands (Cicek et al., 2010). Although the potency of these compounds was apparently rather low, we observed a remarkably high efficacy of I<sub>GABA</sub> enhancement compared to established GABA<sub>A</sub> receptor modulators such as benzodiazepines (e.g. maximal I<sub>GABA</sub> enhancement by the highly potent benzodiazepine site ligand triazolam $E_{\text{max}(\alpha 1\beta 2\gamma 2S)} = 253\pm 12\%$ (data taken from (Khom et al., 2006)) vs. I<sub>GABA</sub> enhancement by Ac-SM $E_{\text{max}(\alpha 1\beta 2\gamma 2S)} = 1187\pm 166$ in this study (Sigel and Baur,
1988)). Here, we analyzed potential subunit-selective effects of the most efficacious compound Ac-SM. Ac-SM positively allosterically modulated \( I_{\text{GABA}} \) through nine major \( \text{GABA}_A \) receptor subtypes with equal potency, but slightly different efficacy (Fig.2A-C). Enhancement of \( I_{\text{GABA}} \) by Ac-SM was only slightly influenced by the \( \alpha \)-subunit isoform incorporated into the channel, but not by the \( \beta \)-subunit isoform and did not depend on the presence of a \( \gamma_{2S} \)-subunit. Based on these observations, we exclude an interaction of Ac-SM with the benzodiazepine binding site (Sigel and Buhr, 1997; Rudolph et al., 1999; Olsen and Sieghart, 2009; Richter et al., 2012; Sigel and Steinmann, 2012). In addition, as Ac-SM efficaciously modulates a broad variety of \( \text{GABA}_A \) receptor subtypes, its profile apparently reminds of \( \text{GABA}_A \) receptor modulators such as barbiturates or neurosteroids. However, whether Ac-SM also shares a common binding site with these compounds or whether Ac-SM interacts with a novel, yet undefined binding site on \( \text{GABA}_A \) receptors remains to be exploited in future studies.

Our \textit{in vivo} experiments revealed a wide spectrum of effects induced by low doses of Ac-SM, which fit to the profile of non-subtype-selective \( \text{GABA}_A \) agonists or positive allosteric modulators (Rudolph et al., 1999; Sieghart, 2000; Olsen and Sieghart, 2009; Möhler, 2011; Sigel and Steinmann, 2012). These effects include reduction of motor activity paralleled by reduced stress-induced hyperthermia. Due to the strong motoric inhibition at higher doses (Fig. 4A) suggesting pronounced sedative effects, potential anxiolytic effects of Ac-SM (Fig. 6A-C) are difficult to assess, however, increased exploration of the open arms of the elevated-plus-maze was observed at low doses not affecting motor activity. The motor activity-independent stress-induced-hyperthermia-test
revealed a marked dose-dependent reduction of stress response (Fig.7), supporting the assumption of anxiolytic effects induced by Ac-SM (Olivier et al., 2003). Furthermore, we observed a dose-dependent increase in PTZ-induced seizure threshold (Fig.8). The long lasting reduction of activity in the home cage suggests a long biological half-life of Ac-SM (Fig.5).

Taken together, our data demonstrate that the recently identified novel GABA_\text{A} receptor modulator Ac-SM enhances I_{GABA} through nine receptor subtypes with similar potency but slightly different efficacies (\(\alpha_{1/2/3} \beta_{2/3/5/6} \gamma_{2S} \sim \alpha_{1/2} \gamma_{2S} > \alpha_{4} \beta_{2} \gamma_{2S}\)). Due to the positive GABA_\text{A} allosteric modulatory effects of Ac-SM (and potentially other cycloartane glycosides, (Cicek et al., 2010)), it is tempting to speculate that this GABA_\text{A} receptor modulation leading to sedation and muscle relaxation contributes to the alleviation of mild to moderate symptoms in menopause reported in clinical studies with preparations from Actaea racemosa L., especially when hot flushes are associated with sleep and mood disturbances (Wuttke et al., 2003; Frei-Kleiner et al., 2005; Osmers et al., 2005; Verhoeven et al., 2005; Newton et al., 2006; Pockaj et al., 2006; Uebelhack et al., 2006; Cheema et al., 2007).

Actaea racemosa L. preparations are currently standardized for their 23-epi-26-deoxyacteatin content (Pepping, 1999), one of the 5 main Actaea triterpenoids (acteatin, 23-epi-26-deoxyacteatin, cimigenol-3-O-\beta-d-xylopyranoside, cimiracemoside C and cimiracemoside F). Significant, however varying amounts of Ac-SM were recently identified in Actaea racemosa L. plant material (0.0137 ± 1.65% bzw. 0.0092 ± 1.97%) as well as in four Actaea preparations (0.004 ± 6.42% - 0.0184 ± 1.92%) that are commercially available in Austria (Cicek et al., 2011). Whether these estimated amounts
of pure Ac-SM are also sufficient to induce anxiolytic, stress-reducing, anticonvulsant and sedative effects in humans, remains, however, to be clarified. Due to the efficacy of Ac-SM and its long biological half-life (Fig.5) cumulative sedative side effects of repetitive intake of Actaea racemosa L. preparations might not be neglectable. In addition, Actaea preparations are consumed in most cases without prescription. As Ac-SM as well as other hitherto unknown components/metabolites of Actaea racemosa L. preparations interact amongst others with various targets in the central nervous system, potential harmful interactions with other psychoactive drugs- in particular CNS-depressant drugs- should be also carefully considered.
Authorship contributions

Participants in research design: Khom S., Schwarzer C. and Hering S.

Conducted experiments: Strommer B., Khom S., Kastenberger I., Schwarzer C.

Contributed new reagents or analytical tools: Cicek SS., Stuppner H.

Performed data analysis: Strommer B., Khom S., Kastenberger I., Schwarzer C.

Wrote or contributed to the writing of the manuscript: Khom S., Schwarzer C., Hering S.
References


Footnotes

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Figure Legends

Figure 1
Structural formula of 23-O-acetylshengmanol 3-O-β-d-xylopyranoside (Ac-SM)

Figure 2
Subtype-dependent modulation of GABA<sub>A</sub> receptors by Ac-SM
Concentration-response curves for the modulation of GABA-induced chloride currents (I<sub>GABA</sub>) through GABA<sub>A</sub> receptors composed of (a) (●) α<sub>1</sub>β<sub>2</sub>γ<sub>S</sub>, (■) α<sub>1</sub>β<sub>2</sub>, (b) (●) α<sub>1</sub>β<sub>2</sub>γ<sub>S</sub>, (□) α<sub>2</sub>β<sub>2</sub>γ<sub>S</sub>, (◇) α<sub>3</sub>β<sub>2</sub>γ<sub>S</sub>, (▼) α<sub>4</sub>β<sub>2</sub>γ<sub>S</sub>, (○) α<sub>5</sub>β<sub>2</sub>γ<sub>S</sub>, (Δ) α<sub>6</sub>β<sub>2</sub>γ<sub>S</sub>, (●) α<sub>1</sub>β<sub>2</sub>γ<sub>S</sub>, and (▲) α<sub>1</sub>β<sub>3</sub>γ<sub>S</sub> subunits by Ac-SM. Each data point represents the mean±SEM of at least five oocytes from two different batches. (d) Typical traces illustrating I<sub>GABA</sub> potentiation through α<sub>2</sub>β<sub>2</sub>γ<sub>S</sub> GABA<sub>A</sub> by the indicated concentration of Ac-SM.

Figure 3
Ac-SM shifts the GABA concentration-response curve towards higher GABA sensitivity
GABA concentration-response curves for α<sub>1</sub>β<sub>3</sub> GABA<sub>A</sub> receptors in the absence (control, ◆) and in the presence of 300µM Ac-SM (◇) are compared. Each data point represents the mean±SEM from at least three oocytes from two different oocyte batches.

Figure 4 Ac-SM dose-dependently reduces locomotor activity in the OF test
Bars indicate in (a) the total distance travelled, in (b) the time spent in the centre, in (c) the number of entries to the centre and in (d) the distance travelled in the centre (% of the total distance) of mice treated with the indicated dose Ac-SM (mg/kg bodyweight; grey bars) compared to vehicle-treated control littermates (white bars). Bars always represent means±SEM, number of animals per group are given in the respective bar. (*) indicates statistically significant differences with \( p<0.05 \), (**) \( p<0.01 \) to control (ANOVA with post-hoc Bonferroni analysis).

**Figure 5**

**Ac-SM reduces locomotor-activity for 12 hours without rebound effect.**

Activity (given as arbitrary activity counts) in the home cage of control (black circles) and Ac-SM-treated (20mg/kg, red circles) mice is compared. The experiment was started at 5.00 pm (=time point 0) lasting for 72h. Light-on phases of the light-dark cycle are highlighted as yellow bars on the x-axis. Each data point represents a mean±SEM from 8 mice.

**Figure 6**

**Ac-SM displays anxiolytic effects in the EPM**

Bars illustrate in (a) the number of open arm (OA) entries in % of the total number of entries, (b) the time spent in the open arms (OA) in % of the total time and in (c) the distance travelled in the OA in % of the total distance after application of the indicated dose in mg/kg bodyweight of Ac-SM (grey bars) in comparison to a saline-treated control group (white bars). Bars represent means±SEM, number of animals per group are given.
in the respective bar. (*) indicates statistically significant differences with $p<0.05$ to control (ANOVA with post-hoc Bonferroni analysis).

**Figure 7**

**Ac-SM dose-dependently reduces stress-induced hyperthermia (SIH)**

Bars indicate SIH ($\Delta T$; °C) 3 hours after application of either control solution (white bars) or Ac-SM at the indicated dose (grey bars). Bars represent means±SEM, number of animals per group are given in the respective bar. (*) indicates statistically significant differences with $p<0.05$, (**) $p<0.01$ to control (ANOVA with post-hoc Bonferroni analysis).

**Figure 8**

**Ac-SM displays anticonvulsant activity.**

Bars indicate changes in seizure threshold upon pentylenetetrazole (PTZ) infusion of the indicated dose of Ac-SM (grey bars) compared to control-treated littermates (white bars). Each bar represents the mean±SEM, the number of tested animals is given in the respective bar. (**) indicates statistically significant differences with $p<0.01$, (***) $p<0.001$ to control (ANOVA with post-hoc Bonferroni analysis).
Table 1

Summary of potencies (EC$_{50}$), efficiencies (maximal potentiation of I$_{GABA}$), Hill-coefficients (n$_H$) and number of experiments of Ac-SM enhancement of GABA$_A$ receptors with different subunit compositions

<table>
<thead>
<tr>
<th>Subunit combination</th>
<th>EC$_{50}$ [µM]</th>
<th>max. Potentiation of I$_{GABA}$ [%]</th>
<th>n$_H$</th>
<th>number of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_1\beta_2\gamma_2S$</td>
<td>47.1 ± 20.9</td>
<td>1187 ± 166</td>
<td>1.0 ± 0.1</td>
<td>6</td>
</tr>
<tr>
<td>$\alpha_2\beta_2\gamma_2S$</td>
<td>39.1 ± 8.4</td>
<td>1454 ± 97</td>
<td>1.7 ± 0.3</td>
<td>7</td>
</tr>
<tr>
<td>$\alpha_3\beta_2\gamma_2S$</td>
<td>47.5 ± 11.7</td>
<td>1174 ± 218</td>
<td>2.0 ± 0.3</td>
<td>4</td>
</tr>
<tr>
<td>$\alpha_4\beta_2\gamma_2S$</td>
<td>46.3 ± 4.7</td>
<td>752 ± 53</td>
<td>1.4 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td>$\alpha_5\beta_2\gamma_2S$</td>
<td>50.9 ± 11.8</td>
<td>1408 ± 87</td>
<td>1.7 ± 0.4</td>
<td>3</td>
</tr>
<tr>
<td>$\alpha_6\beta_2\gamma_2S$</td>
<td>46.0 ± 22.9</td>
<td>1171 ± 274</td>
<td>1.0 ± 0.1</td>
<td>3</td>
</tr>
<tr>
<td>$\alpha_1\beta_2$</td>
<td>45.0 ± 11.4</td>
<td>1277 ± 243</td>
<td>1.5 ± 0.3</td>
<td>5</td>
</tr>
<tr>
<td>$\alpha_1\beta_1\gamma_2S$</td>
<td>43.4 ± 15.9</td>
<td>1069 ± 214</td>
<td>1.6 ± 0.4</td>
<td>6</td>
</tr>
<tr>
<td>$\alpha_1\beta_3\gamma_2S$</td>
<td>35.4 ± 12.3</td>
<td>1247 ± 222</td>
<td>1.7 ± 0.4</td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 5

The graph illustrates the activity counts over time, comparing control (black circles) and Ac-SM 20 mg/kg (red squares) treatments. The x-axis represents the duration in hours, ranging from 0 to 64, and the y-axis shows the activity counts from 0 to 2500. The data points are accompanied by error bars, indicating variability in the measurements.
Figure 6

A

Entries OA; %

Control  0.2  0.6

B

Time OA; %

Control  0.2  0.6

C

Distance OA; %

Control  0.2  0.6
Figure 8

Pentyleneetetrazole: mg/kg

mg/kg

Control 0.2 0.6 2 6 20 60

6 4 4 4 5 4 3

0 35 40 45 50 55 60 65

*** **