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Betulin binds to γ-aminobutyric acid receptors and exerts anticonvulsant action in mice

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ABSTRACT

The lupane type pentacyclic triterpenes: lupeol, betulin, and betulinic acid are widely distributed natural compounds. Recently, pharmaceutical compositions from plant extracts (family Marcgraviaceae) containing betulinic acid, have been patented as anxiolytic remedies. To extend our knowledge of the CNS effects of the triterpenes, we suggest here that the chemically related lupeol, betulin and betulinic acid may interact with the brain neurotransmitter γ-aminobutyric acid (GABA) receptors in vitro and in vivo. Using radioligand receptor-binding assay, we showed that only betulin bound to the $GABA_A$-receptor sites in mice brain in vitro and antagonised the $GABA_A$-receptor antagonist bicuculline-induced seizures in mice after intracisternal and intraperitoneal administration. Neither betulinic acid nor lupeol bound to $GABA_A$ receptor nor did they inhibit bicuculline-induced seizures in vivo. These findings demonstrate for the first time the CNS effects of betulin in vivo, and they also show distinct $GABA_A$-receptor-related properties of lupane type triterpenes. These findings may open new avenues in understanding the central effects of betulin, and they also indicate possibilities for novel drug design on the basis of betulin structure.

1. Introduction

The pentacyclic triterpenes lupeol, betulin and betulinic acid (chemical structures shown in Fig. 1) are compounds widely distributed throughout the plant kingdom. Among them, betulin is the most abundant (for reviews see: Patočka, 2003; Alakurtti et al., 2006). The content of betulin in the outer bark of the white birch varies between 10 and 35% from the total dry weight of the outer bark extract. The content of betulinic acid is 1 to 2%, but lupeol is found only in trace amounts (Patočka, 2003; Alakurtti et al., 2006). Chemically pentacyclic triterpenes share similar structural moieties comprising four six-membered rings and one five-membered ring. Betulin (also known as betulinol, betuline or betulinic alcohol) is a pentacyclic triterpene alcohol with a lupane skeleton (Fig. 1).

In general, studies on the biological activities of lupane derivatives include anti-inflammatory (Zdzisinska et al., 2003; Yamashita et al., 2002), antiviral (Gong et al., 2005; San et al., 1998), anticancer, antimalarial, and antifungal action (Zuco et al., 2002; Fulda et al., 1999; Pisha et al., 1995; Patočka, 2003; Alakurtti et al., 2006), but CNS effects of the triterpenes have not been comprehensively examined using in vivo assays. Current literature reveals indications of anxiolytic activity of betulinic acid isolated as a bioactive component from extracts of plants of family Marcgraviaceae (shrubs, small trees and lianas in Tropical and Central America, and West Indies). Also, a pharmaceutical composition containing betulinic acid is patented as a means for prevention or treatment of anxiety (Durst et al., 2002). Recently, anti-anxiety activity of betulinic acid has been described after oral and intraperitoneal (i.p.) administration in mice and rats (Durst et al., 2002). The ability of betulinic acid after i.p. administration to cross the blood-brain barrier in CD-1 mice was clearly shown by HPLC/MS analysis (Udeani et al., 1999) when the serum concentrations of the betulinic acid reached peaks at 0.15–0.23 h. The short absorption half-life (9–14 min) indicated that betulinic acid is easily absorbed after i.p. injection (Udeani et al., 1999).

We hypothesized that betulin and lupeol, as lipophilic molecules, may also penetrate the blood-brain barrier, as already demonstrated for the betulinic acid, and that they may possess CNS activity. Here, we investigated the binding of the betulin, betulinic acid and lupeol to the $GABA_A$ receptor in radioligand binding assay by using labelled [^{3}H] GABA for the GABA site and [^{3}H]flunitrazepam for the benzodiazepine site. We studied the influence of the triterpenes on $GABA_A$-receptor

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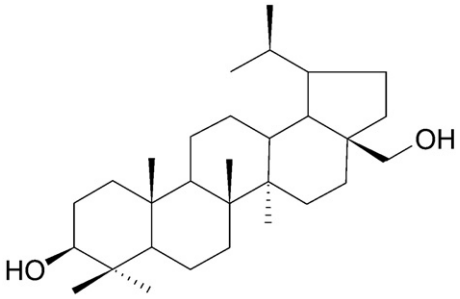

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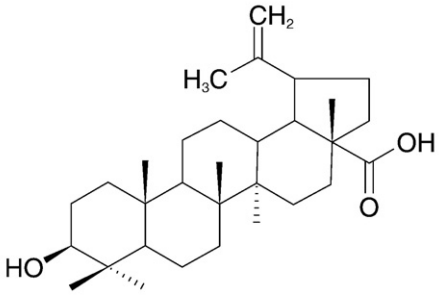

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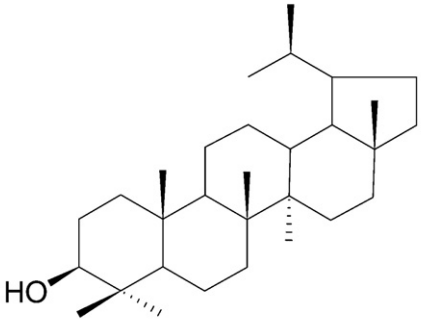

LUPEOL

Fig. 1. Chemical structures of pentacyclic triterpenes: betulin, betulinic acid and lupeol.

antagonist bicuculline-induced seizures in mice after intracisternal (i.c.) and i.p. administration in vivo.

The present studies were designed to determine whether or not betulin, betulinic acid and lupeol may influence $GABA_A$-receptor-mediated processes in vitro and in vivo.

2. Materials and methods

2.1. Materials

Betulin, betulinic acid, lupeol, bicuculline and all other chemicals were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Labelled ligands [^{3}H]GABA (92 Ci/mmol) and [*N*-methyl-^{3}H]flunitrazepam (in text [^{3}H]flunitrazepam (91 Ci/mmol) were purchased from Amersham Biosciences (Uppsala, Sweden).

Triterpenes were dissolved in 100% dimethylsulfoxide (DMSO) to prepare 10 mM stock solutions that were diluted with PBS buffer before the radioligand binding experiments. For the in vivo tests, triterpenes were dissolved in 0.5% w/v Tween-80 in saline and further diluted with isotonic saline up to the required concentration. Bicuculline was dissolved in 0.1 N HCl and diluted with isotonic saline to a final concentration of 0.1 mg/ml, pH 7.

2.2. Animals

Male ICR mice (from the Laboratory Animal Breeding Facility, Riga Stradins University, Riga, Latvia) weighing 23–25 g were housed under standard conditions (21–23 °C, 12 h light–dark cycle) with unlimited access to standard food and water. All experimental procedures were carried out in accordance with guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by Ethics Council of Animal Protection at the Veterinary and Food Service, Riga, Latvia.

2.3. [^{3}H]flunitrazepam binding

For [^{3}H]flunitrazepam binding tissues were prepared as described previously (Cope et al., 2004; Klusa et al., 1990). Briefly, male ICA mice (20–25 g) were decapitated, and their forebrains were rapidly homogenised in 32 volumes (ratio of grams of wet brain tissue to ml of buffer) of ice-cold buffer (50 mM Tris/HCl, pH 7.4). The homogenates were ultracentrifuged at 150 000 ×g for 15 min, and the pellet stored for 30 min at −20 °C. The pellet was resuspended in the same volume of 50 mM Tris/HCl, pH 7.4 buffer and centrifuged at 150 000 ×g for 15 min. This procedure was repeated three times, and, finally, the pellet was resuspended in 50 mM Tris/HCl buffer at a protein concentration of 0.5–0.7 mg/ml. Membrane protein concentration was determined by the method of Bradfod (1976) with bovine serum albumin as a standard. Membranes were stored frozen at −80 °C until use.

For inhibition studies, membranes, supplemented with 3 nM [^{3}H] flunitrazepam and various concentrations of the test drugs, were incubated in 50 mM Tris/HCl buffer for 60 min at 4 °C using the 96-well MultiScreen harvest plates. To define non-specific binding, 100 μM diazepam was used. After incubation, the suspensions were rapidly filtered and washed through Whatman GF/B filters. Filters were subjected to liquid scintillation counting using OptiPhase scintillation cocktail. The amount of bound radioactivity was counted for 1 min with 1450 Microbeta Trilux (Wallac, Finland) liquid scintillation and luminescence counter.

2.4. [^{3}H]GABA binding

Membranes were prepared as described previously (Mehta and Ticku, 2001). Briefly, mice forebrain tissue was homogenised in ice-cold 0.32 M sucrose (pH 7.4), 20 ml/g of tissue, and centrifuged at 140 000 ×g for 30 min at 4 °C to obtain the mitochondrial plus microsomal fraction. This fraction was dispersed in ice-cold distilled deionised water, and homogenised. Then, the suspension was centrifuged at 140 000 ×*g* for 30 min at 4 °C. The resulting pellet was resuspended in ice-cold 50 mM Tris/HCl buffer and centrifuged again at 140 000 ×g for 30 min at 4 °C. This step was repeated two more times. After the final centrifugation, the pellet was resuspended in a small volume of 50 mM Tris/HCl buffer (pH 7.4) and stored frozen at −20 °C overnight. Finally, membranes were thawed and resuspended at 20 ml/g of tissue in 50 mM Tris/HCl buffer and centrifuged at 140 000 ×g for 30 min at 4 °C. The procedure was repeated twice. After that, the pellet was resuspended in the buffer for use in the assay at a protein concentration of 0.5–0.7 mg/ml.

To determine the specific binding, the membranes were incubated with 10 nM [^{3}H]GABA and the various drugs in 50 mM Tris/HCl, 50 mM KCl, pH 7.4 buffer for 60 min at 4 °C. To determine non-specific binding, 100 μM GABA was used. After each incubation, the suspensions were rapidly filtrated and washed through Whatman GF/B filters. Filters were subjected to liquid scintillation counting using OptiPhase scintillation cocktail. The amount of bound radioactivity was counted for 1 min with 1450 Microbeta Trilux (Wallac, Finland) liquid scintillation and luminescence counter.

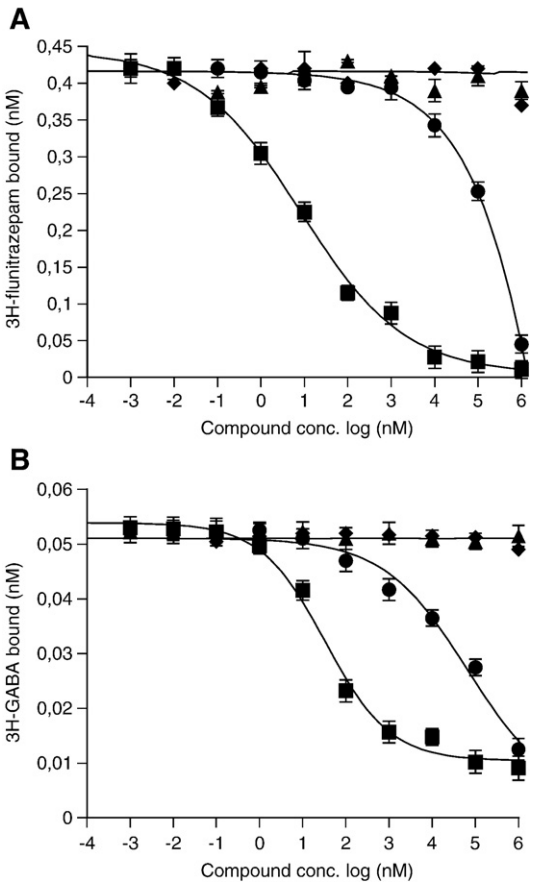

Fig. 2. Binding of triterpenes to the $GABA_A$-receptor benzodiazepine and GABA sites. A. Inhibition of binding of fixed 3 nM concentration of [^{3}H]flunitrazepam by varying concentrations of the non-labelled competing compounds: diazepam (squares), betulin (circles), betulinic acid (triangles) and lupeol (rhombs) to mice forebrain membranes. B. Inhibition of binding of fixed 10 nM concentration of [^{3}H]GABA by varying concentrations of the non-labelled competing compounds: GABA (squares), betulin (circles), betulinic acid (triangles) and lupeol (rhombs) to mice forebrain membranes. Experiments were repeated three times in triplicates.

2.5. Bicuculline-induced seizure threshold test

Seizure threshold was determined according to the method described elsewhere (Devaud et al., 1995). Mice were restrained in a Plexiglas plunger-style mouse restraint (Braintree Scientific, Inc.) for insertion of a 26-gauge needle into the lateral tail vein. The needle was fixed to the tail with a narrow piece of adhesive tape, and the animal was released into the cage to allow free movement. The determination of seizure threshold was made by intravenous (i.v.) infusion (Infusion pump, Model SP100iZ, World Precision Instruments) of bicuculline at a constant rate of 0.5 ml/min and by recording the bicuculline dose that caused the first myoclonic jerk of the head and neck. Seizure threshold was calculated as bicuculline dose per body weight (mg/kg) in the presence or absence of the test compound. The test compounds were administered i.c. or i.p. at 15 min and 30 min before bicuculline infusion, respectively. Each group contained 10–12 animals, with each animal being tested once, and, the observer was blind to groups and treatments.

2.6. Rota-rod test

Rota-rod test (Dunham and Miya, 1957) in mice was used to measure the motor coordination by use of rota-rod apparatus (Model 7600, Ugo Basile, Italy). One day before the experiment, animals were trained on the rota-rod apparatus, and the animals that failed to stay on the rotating (15 rpm) rod for 180 s were excluded from further testing. On the experiment day, triterpenes were injected in the selected mice (n=10 per group) 2 h after the control test. Due to the low solubility of betulinic acid and lupeol, only betulin at different doses in a volume of 10 μl was injected i.c., whereas all three triterpenes under study: betulin, betulinic acid and lupeol were administered i.p. in a volume of 100 μl.

After 15 min following i.c. injection of betulin or vehicle, and 30 min after i.p. administration of the compounds, the mice were placed on the rotating rod, and the time spent on the rota-rod for each mouse was recorded upon 180 s.

2.7. Data analysis

The data were analysed with the GraphPad Prism software for each binding experiment in triplicate, and the mean±S.D. from 3 independent experiments was calculated. Statistical significance for seizure threshold was calculated by one-way ANOVA with Dunnett's Multiple Comparison Test as post-hoc analysis. p-values less than 0.05 were considered statistically significant.

3. Results

3.1. $GABA_A$-receptor binding

Betulin, lupeol and betulinic acid in the concentration range from 0.1 nM to 1 mM were tested for binding to the mice forebrain membrane $GABA_A$ receptors in vitro using the radioligand binding assay. $GABA_A$-receptor GABA-site ligand [^{3}H]GABA and selective benzodiazepine site agonist — [^{3}H]flunitrazepam were used as labelled ligands. As seen from Fig. 2A, betulinic acid and lupeol did not displace the [^{3}H]flunitrazepam from the binding site, whereas betulin slightly competed for the binding to the benzodiazepine sites. In view of the DMSO inhibiting effect on binding of the [^{3}H] flunitrazepam, however, the effect of betulin was not considered statistically significant. Indeed, solvent binding controls revealed that DMSO concentrations above 1% interfere with binding of labelled [^{3}H] flunitrazepam (Fig. 3). The reference drug diazepam almost completely displaced the label (Fig. 2A). Non-specific binding was low, in the range of 1–4% of the total binding, and the calculated diazepam inhibition constant (K_i) averaged 6±2 nM, as estimated from three independent experiments.

[^{3}H]GABA binding was less influenced by DMSO. As seen from Fig. 3, the DMSO effect was observed starting at 20% DMSO.

As seen from Fig. 2B, betulin bound to the $GABA_A$ receptors with an average K_i value of 64±5 μM, whereas betulinic acid and lupeol did

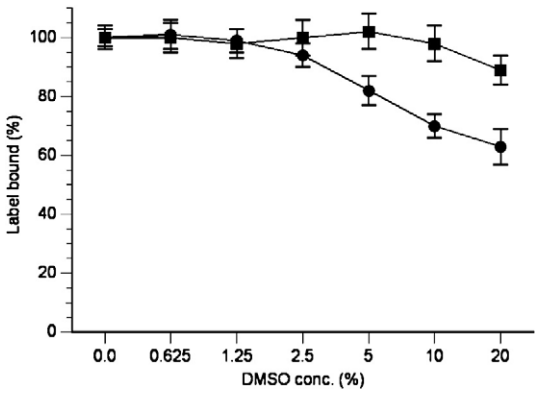

Fig. 3. Effect of DMSO on [3H]flunitrazepam (cycles) and [3H]GABA (squares) binding to mice forebrain membranes. Experiments were repeated three times in triplicates.

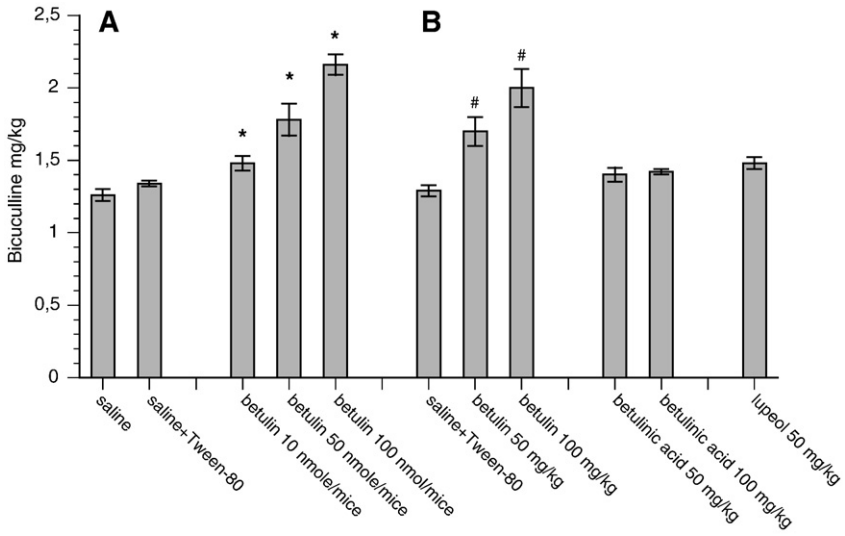

Fig. 4. Inhibition of bicuculline-induced seizures by triterpenes. A. Betulin was administered i.c. 10 min before the bicuculline i.v. infusion. B. Betulin, betulinic acid and lupeol were administered i.p. 30 min before the bicuculline i.v. infusion. The values are mean ± S.D. ($n = 10$). * $p \leq 0.05$ vs. vehicle control after i.c. administration; # $p \leq 0.05$ vs. vehicle control after i.p. administration, one-way ANOVA with Dunnett's Multiple Comparison Test.

not show specific displacement of the label. Non-labelled GABA was used as reference drug, showing a characteristic competition curve with approximately 15% non-specific binding and an average K_i value of 18 ± 4 nM.

3.2. Anticonvulsant action

The i.v. dose of bicuculline that induced the first myoclonic jerks was found to be approximately 1.2 mg/kg (Fig. 4).

Since betulin was dissolved in saline with the help of Tween-80, the data obtained with this solvent are shown as controls. As seen in Fig. 4, Tween-80 by itself did not influence the bicuculline effect. It was not possible to dissolve betulinic acid and lupeol in saline even with the help of Tween-80, so these substances were not injected i.c. Hence, the data shown in Fig. 4A include the only results from betulin administered i.c. 15 min prior to bicuculline. I.c. administration of betulin at doses of 10 nmol/mice, 50 nmol/mice and 100 nmol/mice significantly antagonised the bicuculline effect, which manifested as the increased bicuculline dose necessary to induce the first myoclonic jerks.

Fig. 4B shows that i.p. administration of betulin at doses of 50 mg/kg and 100 mg/kg 30 min prior to bicuculline i.v. infusion significantly inhibited the bicuculline effect. Neither betulinic acid nor lupeol influenced the convulsant action of bicuculline.

3.3. Influence on muscle tone and coordination

The data shown in Table 1 demonstrate that betulin after its i.c. administration did not cause any influence on mice muscle tone and coordination in the rota-rod assay. Similarly, after i.p. administration, neither betulin nor betulinic acid and lupeol exerted any muscle relaxing activity in mice. Vehicle in both experiments was injected in the volume corresponding to the administration route.

Table 1
Effect of lupane triterpenes on mice behaviour in rota-rod test

Compound	Dose	Administration route	Time spent on rod (s)
Saline+Tween-80		i.c.	179 ± 1.0
Betulin	10 nmol	i.c.	179.75 ± 0.25
Betulin	50 nmol	i.c.	179.5 ± 0.29
Betulin	100 nmol	i.c.	180 ± 0.0
Saline+Tween-80		i.p.	179 ± 0.58
Betulin	50 mg/kg	i.p.	179.5 ± 0.5
Betulin	100 mg/kg	i.p.	179.25 ± 0.48
Betulinic acid	50 mg/kg	i.p.	179 ± 1.0
Betulinic acid	100 mg/kg	i.p.	179 ± 1.0
Lupeol	50 mg/kg	i.p.	179.5 ± 0.5

Compounds were administered 15 min i.c. and 30 min i.p. before the rota-rod test. The values are expressed in s as mean ± S.D., ($n = 10$).

4. Discussion

For the first time, we demonstrate the distinct $GABA_A$-receptor-related properties of lupane type triterpenes in vivo and in vitro. Particular interest can be paid to our findings that betulin competed with [^{3}H]GABA for binding to the corresponding sites on the $GABA_A$ receptor, whereas betulinic acid and lupeol did not show any binding affinity. We also demonstrate the different DMSO effects on the [^{3}H] flunitrazepine and [^{3}H]GABA binding. Previously, the decreased GABA binding (80% of the basal binding) in the presence of 1% DMSO was found by using non-tritiated label [^{32}S]TBPS (a convulsant site ligand of $GABA_A$ receptors) as well, (Holopainen et al., 2001), whereas 0.1% DMSO did not produce a considerable reduction of GABA-mediated receptor-gated currents (Wexler et al., 1998).

In our present study, GABAergic activity of betulin was found in vivo by using bicuculline, a specific antagonist of the $GABA_A$-receptor GABA site, as a convulsant drug. This is in good agreement with the GABA-binding potency of betulin in vitro. For the first time, we demonstrated here, that betulin possesses an anticonvulsant action after both i.c. and i.p. injections in mice. Since betulin, following its central and peripheral administrations, showed antagonistic action against bicuculline, we may suggest a penetration of betulin into the mice brain and its direct binding to the $GABA_A$-receptor GABA site. The observation that betulinic acid and lupeol did not show anticonvulsant activity is in good agreement with our radioligand binding data. Moreover, that result is in good line with the earlier published data (Zhu et al., 1996), that betulinic acid does not bind to $GABA_A$ receptor; no data on betulin binding was found.

We may also suggest, that the studied triterpenes have no influence on muscle tone and coordination at a dose range up to 100 nmol/mouse i.c. or 100 mg/kg i.p.

In summary, the present study provides the first experimental evidence for the ability of betulin to influence $GABA_A$-receptor-mediated signalling pathways. Although betulinic acid and lupeol are chemically closely-related to betulin, they neither bound to the $GABA_A$ receptor nor inhibited bicuculine-induced seizures in mice.

It is hoped that these findings will lead to a better understanding of the influence of betulin on the CNS and a clarification of mechanisms

of the pharmacological action of betulin and that they will promote the use of betulin as a model compound for novel drug design based on bioactive plant substances.

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