Interactions of valerian extracts and a fixed valerian–hop extract combination with adenosine receptors

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Abstract

Phytopharmaceuticals and dietary supplements containing valerian are used as mild sleep-inducing agents. An in vitro radioligand binding assay at A1 and A2A adenosine receptors (ARs) was conducted with a fixed extract combination of valerian and hop (Ze 91019) to investigate a possible mechanism for the pharmacological activity of the extract. Component extracts of valerian and hop were also individually investigated. The fixed combination Ze 91019 as well as the valerian extracts therein exhibited selective affinity to A1 ARs (Ki = 0.15–0.37 mg/mL vs [3H]CCPA). The same extracts exhibited partial agonist activity at the A1 adenosine receptor as indicated by a lower degree of stimulation of [35S]GTPγS binding in membrane preparations of CHO-hA1 cells as compared to the full A1 AR agonist N6-cyclopentyladenosine (CPA). In addition valerian extract inhibited cAMP accumulation in CHO-hA1 cell membranes. The partial agonistic activity at A1 ARs may thus play a role in the sleep inducing effect of Ze 91019 and the valerian extract therein.

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Introduction

The physiological role of the purine nucleoside adenosine has been under investigation since the beginning of the 20th century [1]. Adenosine, as endogenous ligand released from various body cells,
interacts with G-protein coupled cell surface receptors (P1-receptors; adenosine receptors, ARs) that have been subdivided into four subtypes, A1, A2A, A2B and A3 ARs, on the basis of pharmacological studies. Also, the various ARs have recently been cloned from several species [2]. The pharmacological effects resulting from ligand-receptor interaction depend upon the organ and tissue type and the receptor subtype being targeted. P1-purinergic receptors are modulating the activity of adenylate cyclase, A1 and A3 AR activation leads to an inhibition while A2A and A2B AR activation leads to a stimulation of adenylate cyclase [2]. Coupling to other second messenger systems has been described, e.g. stimulation of phospholipase C and potassium channels by A1 ARs. AR activation may, thus, affect the heart and blood vessels, the lungs, the central and peripheral nervous system, and the kidneys, among other organs. Consequently, diverse physiological functions may be modulated that include induction of sedation, vasodilation, suppression of cardiac rate and contractility, neurotransmitter release, inhibition of platelet aggregation and lipolysis [1].

In spite of the physiological significance of ARs, there has been a limited progress in adenosine therapeutics due to the non-localized action of adenosine, scarcity of receptor selective ligands and lack of knowledge of disease states that involve purinergic etiology [3]. One of the main directions of research has been to target the cardiovascular system and hypertension. The recent development of highly potent and subtype-selective AR ligands has rekindled the desire to identify new targets for adenosine-based therapeutical applications and to develop new pharmacological tools for further characterization. Adenosine plays an important role as an inhibitory CNS neuromodulator related to the highly specific coupling to A1 and A2A ARs. Therefore, one of the pharmacological targets includes the central nervous system, with agonists (e.g. cyclohexyladenosine or phenylisopropyladenosine) being sedatives and anticonvulsants, and antagonists (e.g. caffeine and theophylline) being stimulants [3–6]. An indirect modulation of adenosine function, through allosteric effects or control of adenosine level, may also be involved in the mechanism of action of therapeutically investigated entities. The finding that A1 and A2A receptors are highly expressed in the brain and GABAergic neurons, respectively [2], and that both receptors are involved in sleep promotion and regulation [7] support the CNS targeting approach of adenosine therapeutics. Of special interest is the development of partial agonists bearing several promising advantages over full agonists; partial agonists may exhibit a wider therapeutic use due to their less hypotensive side-effects on the cardiovascular system, they induce less receptor down-regulation and may show more subtype-selectivity [8,9]. Interactions of naturally occurring compounds belonging to such phytochemical classes as flavonoids, alkaloids and lactams, have recently been reported with various degrees of binding affinities to the main AR subtypes [10,11]. One of the most studied phytopharmaceuticals targeting the CNS is valerian, which has been used as a mild sleep aid for centuries, alone or in combination with other herbal remedies, such as hop, lemon balm and passionflower [12]. The action of valerian on the CNS might be due in part to GABA involvement through a number of mechanisms [13]. The available data, however, do not preclude the involvement of other targets/mechanisms, especially if valerian is used in combination with other herbs. For example, a hydroalcoholic extract of valerian was found to bind to A1 adenosine receptors in a rat brain cortical membrane preparation and to competitively displace the radiolabeled A1 ligand, [3H]N6-cyclohexyladenosine, in a dose-dependent pattern [14]. Also, it has recently been reported that a lignan isolated from valerian root showed partial agonist activity at A1 adenosine receptors [11,15]. Caffeine, on the other hand, acts as a non-selective antagonist at ARs exhibiting CNS stimulatory effects [3]. In an attempt to further investigate the mechanism of action of a valerian-based phytopharmaceutical or dietary supplement, we have screened it against ARs of the A1 and A2A subtypes, which are the predominant subtypes
in brain. The herbal product (Ze 91019), which contains a fixed combination of valerian and hop extracts (25:6, w/w) exhibited a partial agonist activity at the A₁ AR subtype. Results of our investigation are presented herein.

Materials and methods

All chemicals and cell culture media were purchased from Sigma (Taufkirchen, Germany), unless otherwise noted.

Extracts

Valerian dry extracts (VE-1, VE-2) were prepared from dried roots of Valeriana officinalis L. (Valerianae rhizoma Ph. Eur.), and hop dried extract (HE) was prepared from dried cones of Humulus lupulus L. (Humuli lupuli strobuli Ph. Eur.) by mazeration with methanol/water 45% (w/w) at ambient temperature for 2 h with stirring. Then the extracts were separated from the plant material by decanting. After concentration by vacuum evaporation the extracts were spray-dried with the addition of 25% of maltodextrin (Ph. Eur. quality) for valerian, and 30% for hop, respectively, relative to the total dry mass. The drug:extract ratio was 4–6:1 for the valerian extract and 5–7:1 for the hop extract. The valerian extract VE-1 (batch no. 011540) contained 0.388% of valerenic acids (0.173% of valerenic acid, 0.215% of acetoxyvalerenic acid), VE-2 (batch no. 012270) contained 0.370% of valerenic acids (0.198% of valerenic acid, 0.172% of acetoxyvalerenic acid) as determined by HPLC–DAD. The extracts were free of valepotriates. These were eliminated by the extraction process since 45% of methanol is too hydrophilic to extract relevant amounts of essential oils of valerian. The hop extract (HE, batch no. 012450) contained 0.479% of flavonoids (photometrically determined); it contained no hop bitter acids due to the extraction process using hydrophilic solvents. In contrast, the hydrophilic amino acids, in discussion as new lead compounds for both extracts, as well as the lignans, were extracted completely with 45% methanol/water. Ze 91019 is a fixed combination consisting of 250 mg of the valerian extract and 60 mg of the hop extract per tablet. DMSO was used to dissolve the dry extracts for utilization in the bioassays.

Radioligand binding assays

The extracts were investigated in radioligand binding assays at A₁ ARs of rat brain cortical membranes using the A₁ selective radioligand [³H]2-chloro-N⁶-cyclopentyladenosine ([³H]CCPA, 1.110 TBq/mmol, Amersham, Germany), and at A₂A AR of rat striatal membranes using the A₂A-selective radioligand [³H]3-(3-hydroxypropyl)-7-methyl-8-(m-methoxystyryl)-1-propargylxanthine ([³H]MSX-2, 3.145 TBq/mmol, custom-labeled by Amersham, Germany). The binding assays were performed as previously described [16,17]. Frozen rat brains were obtained from Pel Freez®, Rogers, Arkansas, USA, and thawed at 4 °C. Frontal cortex was dissected as A₁ AR source. Right and left striata were dissected for A₂A AR studies. Tissues were homogenized in 50 mM TRIS–HCl buffer pH 7.4. Membrane fractions were purified by a series of centrifugation steps as described [18,19]. Final protein pellets were resuspended in 50 mM TRIS–HCl buffer pH 7.4 and stored in aliquots (at a concentration of ca. 4 mg/mL) at −80 °C until used. Protein concentration
was determined by the method of Bradford, using a BIORad assay kit. Before determination, protein was washed in HEPES–NaOH buffer 10 mM, pH 7.4, to prevent interactions of buffer and the reagents used for colorimetric analysis of the protein content. Bovine serum albumin was used as reference standard. Membranes were preincubated with 0.2 I.U./mL of adenosine deaminase in order to remove endogenous adenosine. The extracts were dissolved in DMSO. A final concentration of 2.5% of DMSO was used in the assays. Inhibition curves were determined using 6–7 different concentrations of test compounds or extracts spanning 3 orders of magnitude. At least three separate experiments were performed each in triplicate, unless otherwise noted. Radioligand binding to rat brain cortical membranes was carried out in TRIS–HCl buffer 50 mM, pH 7.4. Assays were performed incubating the mixtures on a shaking water-bath at 23 °C for 90 min. Nonspecific binding was defined using 10 μM of 2-chloroadenosine and amounted to less than 5% of total binding. [3H]CCPA was used in a final concentration of 0.5 nM. Protein (ca. 70 μg per tube containing a final volume of 1 mL) was added to start the reaction. Incubation was terminated by rapid filtration using a Brandel 48-channel cell harvester (Brandel, Gaithersburg, Maryland, USA) through Whatman GF/B glass fiber filters, which had been presoaked in rinse buffer (TRIS–HCl 50 mM, pH 7.4). Filters were rinsed three times with 2 mL of TRIS–HCl buffer 50 mM, pH 7.4, 4 °C.

Radioligand binding to rat brain striatal membranes was carried out in TRIS–HCl buffer 50 mM, pH 7.4. Assays were performed incubating the mixtures on a shaking water-bath at 23 °C for 30 min. Nonspecific binding was defined using 50 μM NECA and amounted to less than 25% of total binding. Total binding was determined in the presence of 2.5% DMSO. [3H]MSX-2 was used in a concentration of 1 nM. Ca. 70 μg of protein per tube (containing a final volume of 1 mL) was added to start the reaction. Termination of the incubation was performed by rapid filtration through GF/B glass fiber filters, presoaked in 0.5% aqueous polyethylenimine solution for 45 min, using a Brandel 48 channel cell harvester. Filters were washed three times with 2 mL of ice-cold TRIS–HCl buffer 50 mM, pH 7.4.

Radioactivity of the punched-out wet filters was counted after 9 h of preincubation with 3 mL of Ultima Gold scintillation cocktail (Canberra Packard, Dreieich, Germany).

Functional assays

[^3S]GTPγS binding assay

Rat brain cortical membranes (7 μg per tube), or membrane preparations of CHO-hA1 cells (5 μg per tube), respectively, were incubated with 0.1–0.5 nM[^3S]GTPγS (46.3 TBq/mmol, NEN) in a total volume of 200 μL in 50 mM TRIS–HCl buffer pH 7.4 containing 1 mM EDTA, 5 mM MgCl2, 1 mM dithiothreitol, 10 μM GDP, 100 mM NaCl, 2 I.U./mL adenosine deaminase (ADA), 0.5% bovine serum albumin, and extract or test compound, according to Lorenzen et al. [20]. Non-specific binding was determined with 10 μM of unlabeled GTPγS. Incubations were terminated after 45 min at 25 °C by the addition of 1 mL of ice-cold buffer containing 50 mM TRIS–HCl pH 7.4 and 5 mM MgCl2, and rapid filtration through GF/B filters (Whatman) on a Brandel cell harvester, followed by two washing steps with cold buffer (2 mL each). Radioactivity on the filters was measured by liquid scintillation counting after transferring the punched out filters into 2 mL of scintillation cocktail (Ultima Gold, Canberra Packard, Dreieich, Germany).
Cyclic AMP accumulation

Culture flasks with confluent grown cells, were washed with PBS buffer and cells were detached with a cell scraper. A membrane preparation of the cells was obtained according to the method described below. The P1 membrane fraction was resuspended in TRIS-buffer 50 mM, pH 7.4 supplemented with 4 mM EDTA, 10 mM MgCl₂ and 5 μM GTP. The mixture was preincubated for 10 min at 37 °C with 2 IU adenosine deaminase to remove endogenous adenosine. Compounds dissolved in DMSO were diluted in TRIS-buffer 50 mM, pH 7.4, containing EDTA (4 mM), MgCl₂ (10 mM), GTP (5 μM), forskolin (10 μM) and ATP (1 mM), and incubated for 10 min at 37 °C with 100 μL of the membrane suspension. Incubation was terminated by rapidly heating to 96 °C for 2 min. The tubes were cooled down to room temperature and centrifuged at 2,000 g, 10 min, 4 °C, using an Allegra 21R centrifuge (Beckman Coulter, Germany). The supernatant (100 μL) was assayed in a charcoal adsorption-assay using a commercially available kit (Amersham, Germany) to determine cAMP accumulation. Each experiment was carried out in duplicate up to three times.

Cell culture

CHO cells stably transfected with the human A₁ AR (CHO-hA₁) were grown adherently and maintained in Dulbecco’s Modified Eagles Medium F12, supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamate (2 mM) at 37 °C, 5% CO₂. Cells were grown to confluence and subcultured twice a week in a ratio of 1:5 or 1:20. For binding assays, culture medium was removed, cells were washed with PBS and kept frozen at −80 °C until membrane preparation following the procedure described by Klotz et al. [21]. For cAMP assays, membranes were immediately used.

Data analysis

Data were analyzed using GRAPH PAD PRISM Version 3.0 (San Diego, CA, USA). For non-linear regression analysis, the Cheng Prusoff equation and Kᵤ values of 0.5 nM for [³H]CCPA and 8 nM for

<table>
<thead>
<tr>
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<th>A₁ Kᵤ (mg/mL)</th>
<th>A₂A Kᵤ (mg/mL)</th>
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<tbody>
<tr>
<td></td>
<td>rat brain cortical membranes vs. [³H]CCPA</td>
<td>striatal membranes vs. [³H]MSX-2</td>
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<tr>
<td>VE-1</td>
<td>0.15 ± 0.001</td>
<td>2.20 ± 0.60</td>
</tr>
<tr>
<td>VE-2</td>
<td>0.19 ± 0.08</td>
<td>n.d.</td>
</tr>
<tr>
<td>HE</td>
<td>0.13 ± 0.07d</td>
<td>&gt;&gt; 0.3</td>
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<tr>
<td>Ze 91019</td>
<td>0.37 ± 0.16c,d</td>
<td>&gt; 2.5</td>
</tr>
<tr>
<td>caffeine</td>
<td>0.0046 ± 0.0006</td>
<td>0.0063 ± 0.0016</td>
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a n.d. = not determined.
b 9 ± 4% inhibition at a concentration of 0.3 mg/mL.
c 20 ± 7% inhibition at a concentration of 2.5 mg/mL.
d Kᵤ values were statistically not significantly different from each other (p > 0.05).
e Values were significantly different from each other (p < 0.01).
[3H]MSX-2 were used to calculate $K_i$ values from IC$_{50}$ values. Functional assays were analyzed using sigmoidal dose response as nonlinear curve regression analysis. For statistical comparison between groups, data were subjected to analysis of variance followed by unpaired $t$-test.

Results

Affinities for $A_1$ and $A_{2A}$ receptors were determined in radioligand binding assays at rat brain cortical membranes vs [3H]CCPA and in rat brain striatal membranes vs [3H]MSX-2, respectively. Individual valerian and hop extracts were tested alongside of the Ze91019 extract. All extracts exhibited affinity to $A_1$ ARs in a similar concentration range ($K_i$ values 0.15–0.37 mg/mL). The extracts were significantly less potent at $A_{2A}$ ARs, e.g. the valerian extract was 15-fold more potent at $A_1$ ($K_i = 0.15$ mg/mL) than at $A_{2A}$ ARs ($K_i = 2.20$ mg/mL) (see Table 1 and Fig. 1).

Fig. 1. Adenosine receptor binding displacement curves of valerian and hop extracts. (A, C, D) $A_1$: Displacement of [3H]CCPA from rat brain cortical membranes. (B) $A_{2A}$: Competition of valerian root dry extract 1 (VE-1) with the $A_{2A}$-selective antagonist [3H]MSX-2 at rat brain striatal membranes.
In order to investigate whether valerian extract acts as an agonist or an antagonist at A1ARs, its effect on [35S]GTPγS binding to rat brain cortical membranes was measured (Fig. 2). The tested valerian extract (VE-1) exhibited a concentration-dependent stimulation of [35S]GTPγS binding giving a maximal effect of 38% over basal with an EC50 value of 0.87 mg/mL. Essentially the same effect was observed in [35S]GTPγS binding studies at human recombinant A1 ARs (Table 2 and Fig. 3). VE-1, VE-2, and Ze 91019 dose-dependently stimulated binding of [35S]GTPγS, with EC50 values of 0.85, 1.20, and 0.86 mg/mL, respectively, while the pure hop extract (HE) had no effect on [35S]GTPγS binding. The maximal effects of the stimulation of [35S]GTPγS binding by the extracts are shown in Fig. 4. The extracts exhibited a lower degree of maximal stimulation as compared to the full A1 AR agonist N6-cyclopentyladenosine (CPA).

Fig. 2. Stimulation of [35S]GTPγS binding induced by increasing concentrations of valerian extract (VE-1) to rat brain cortical membranes EC50 = 0.87 ± 0.32 mg/mL, the maximal stimulation amounted to 38 ± 3%.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Stimulation of [35S]GTPγS binding and inhibition of adenylate cyclase in membrane preparations derived from recombinant human A1 adenosine receptors expressed in Chinese hamster ovary (CHO) cells</th>
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<tr>
<td></td>
<td>Stimulation of [35S]GTPγS binding to CHO-hA1 cell membranes EC50 ± SEM</td>
</tr>
<tr>
<td>VE-1</td>
<td>0.85 ± 0.05 mg/mL</td>
</tr>
<tr>
<td>VE-2</td>
<td>1.20 ± 0.80 mg/mL</td>
</tr>
<tr>
<td>HE</td>
<td>No stimulation</td>
</tr>
<tr>
<td>Ze 91019</td>
<td>0.86 ± 0.33 mg/mL</td>
</tr>
<tr>
<td>CPA (agonist)</td>
<td>0.017 ± 0.002 μM</td>
</tr>
</tbody>
</table>

a n.d. = not determined.
A₁ ARs are negatively coupled to adenylate cyclase as a second messenger system. Inhibition of forskolin-stimulated cAMP accumulation by VE-1 and the A₁ agonist CPA was determined in CHO cells expressing the human A₁ AR. The agonist CPA and VE-1 both dose-dependently inhibited cAMP accumulation.

![Graph showing % specific binding of [³²S]GTPγS stimulation at membranes prepared from human recombinant A₁ adenosine receptors.](image)

**Fig. 3.** [³²S]GTPγS binding study at recombinant human A₁ adenosine receptors.

![Graph showing % stimulation of [³²S]GTPγS binding over basal (± 100%)](image)

**Fig. 4.** Maximal effect of [³²S]GTPγS stimulation at membranes prepared from human recombinant A₁ adenosine receptors (CHO-hA₁ cell membranes). Compound (agonist CPA) and extracts were all tested at a concentration that gave the maximal effect. Stimulation by VE-2 and Ze91019 was not significantly different in comparison with VE-1 (p > 0.05); Results of hop extract (HE) and the full agonist CPA were significantly different ( **p < 0.01) from VE-1.
accumulation (Fig. 5); and both exhibited IC$_{50}$ values that were consonant with their K$_i$ values from radioligand binding and with EC$_{50}$ values from $[^{35}\text{S}]$GTP$\gamma$S binding studies.

**Discussion**

The central nervous system depressing activity of valerian has been investigated from different perspectives and there is mounting evidence to support such an activity. Although the effect is believed to be mediated mainly through GABA interactions, the involvement of other mechanisms cannot be excluded [14]. In this investigation, the valerian-hop extract Ze 91019 was tested for its activity at the ARs alongside of valerian and hop extracts prepared from the same plant source by extraction with a hydrophilic solvent (45% methanol/water). When tested against the selective radioligands $[^{3}\text{H}]$CCPA (for A$_1$ARs) and $[^{3}\text{H}]$MSX-2 (for A$_2\text{A}$ARs), the valerian extracts exhibited 15-fold higher affinity towards the A$_1$AR. There was no significant difference in affinity between two different batches of valerian extract demonstrating the stability of the manufacturing procedure. In order to investigate whether the extracts possessed agonistic or antagonistic activities, $[^{35}\text{S}]$GTP$\gamma$S binding studies were conducted using rat cortical membranes as well as recombinant human A$_1$ARs expressed in CHO cells. The extracts were able to stimulate binding of $[^{35}\text{S}]$GTP$\gamma$S indicating agonism. However, in comparison with the full A$_1$AR agonist CPA, the maximal stimulation was lower. Consequently, the fixed combination and the valerian extract therein can be considered as partial agonists at the A$_1$AR. The hop extract alone had no effects on $[^{35}\text{S}]$GTP$\gamma$S binding. Thus, hop extract appears to be antagonistic at A$_1$ ARs. However, the use of hop as a sleeping aid and for restlessness and anxiety has been documented

![Graph](image_url)
It can thus be speculated that hop may be active through alternative mechanisms involving such targets as the GABA receptor [24, 25]. Functional assays were performed in cells expressing human A₁ ARs. The tested valerian extract was found to inhibit adenylate cyclase and thus clearly acts as an agonist at A₁ ARs. The current results for valerian extract and a combination of valerian and hop extracts support their use as herbal sleep aids. They are also in accordance with those obtained with other valerian extracts containing a glycoside derivative of the lignan olivil that may be involved in mediating the CNS sedative activity of the herbal product among other components [11, 14]. It is important to emphasize that the exhibited effect of Ze 91019 may be neither qualitatively nor quantitatively similar to those of other valerian products due to the difference in procedures adopted by each manufacturer. Consequently, it may be a feasible next step to develop an analytical method for olivil lignans and to correlate the lignan content in various valerian preparations with their A₁AR activity. If such a correlation exists, the first objective analytical marker may become available for valerian products. Also, since in vitro effects are not always expected to reproduce well in vivo, a possible approach to further investigate the A₁AR mediated mechanism of action of VE-1 and Ze 91019 is through the reversal of caffeine stimulation when administered to human volunteers. The rationale and findings of such an approach will be discussed in another report.

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