BIOCHEMICAL PHARMACOLOGY 73 (2007) 248–258

available at www.sciencedirect.com

ScienceDirect

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

# Interaction of valerian extracts of different polarity with adenosine receptors: Identification of isovaltrate as an inverse agonist at $A_1$ receptors

*Svenja K. Lacher* [a], *Ralf Mayer* [a], *Kathrin Sichardt* [b], *Karen Nieber* [b], *Christa E. Müller* [a,*]

[a] *Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, Bonn, Germany*
[b] *Institute of Pharmacy, Pharmacology for Nature Sciences, University Leipzig, Leipzig, Germany*

ARTICLE INFO

*Article history:*
Received 4 August 2006
Accepted 28 September 2006

*Keywords:*
Valerian
Adenosine receptors
Bioassay-guided fractionation
Isovaltrate
$A_1$ antagonist
Inverse agonist

ABSTRACT

A series of extracts of valerian roots (*Valeriana officinalis* L.) was prepared with solvents of different polarity. Polar as well as nonpolar extracts were found to interact with adenosine $A_1$ receptors. While polar extracts activated $A_1$ receptors (partial agonistic activity), nonpolar extracts showed antagonistic or inverse agonistic activity at $A_1$ receptors, as demonstrated by GTPγS binding assays at human recombinant $A_1$ receptors stably expressed in Chinese hamster ovary (CHO) cells. Guided by radioligand binding assays, fractionation of a lipophilic petroleum ether:diethyl ether (1:1) extract led to the isolation of isovaltrate, which was characterized as a potent, highly efficacious inverse agonist at adenosine $A_1$ receptors ($K_i$ rat $A_1$: 2.05 μM). In experiments at rat brain slices measuring post-synaptic potentials (PSPs) in cortical neurons, isovaltrate at least partly reversed the reduction in the PSPs induced by the adenosine $A_1$ receptor agonist $N^6$-cyclopentyladenosine (CPA). Isovaltrate may serve as a new lead structure for the development of inverse agonists at adenosine $A_1$ receptors. The common use of hydrophilic, but not lipophilic valerian extracts as mild sleep-inducing agents is consistent with the opposite actions of hydrophilic and lipophilic extracts on adenosine receptors.



## 1. Introduction

Extracts of the roots of *Valeriana officinalis* L. (valerian) are popular phyto-pharmaceuticals playing an important role especially in Europe as a frequently used mild sedative agents [1–4]. Anticonvulsive and neuroprotective effects have also been reported [5]. Balduini and Cattabeni were the first to describe that a hydroalcoholic valerian extract exhibited affinity for rat adenosine $A_1$ receptors, and the authors hypothesized that this interaction might contribute to the observed pharmacological in vivo effects [6]. In a subsequent study, we confirmed the affinity of an aqueous-methanolic (45%) extract of valerian for rat brain adenosine $A_1$ receptors, and found that the extract was much less potent at the adenosine $A_{2A}$ receptor subtype [7]. In addition, we demonstrated that the valerian extract also bound to *human* adenosine $A_1$ receptors. In functional experiments we could show that the extract exhibited (partial) agonistic activity [7,8].

* *Corresponding author at*: Institute of Pharmaceutical Chemistry, Pharmaceutical Sciences Bonn, University of Bonn, An der Immenburg 4, D-53121 Bonn, Germany. Tel.: +49 228 73 2301; fax: +49 228 73 2567.
E-mail address: christa.mueller@uni-bonn.de (C.E. Müller).

*Abbreviations:* CHO, Chinese hamster ovary; PSPs, post-synaptic potentials; ADA, adenosine deaminase; CCPA, 2-chloro-$N^6$-cyclopentyladenosine; MSX-2, 3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine; DTT, dithiothreitol; ACSF, artificial cerebrospinal fluid; CPA, $N^6$-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine

0006-2952/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2006.09.029

Brattström and colleagues performed in vivo studies in healthy volunteers showing that a combination of valerian and hops extracts counteracted the central stimulatory effects of caffeine as analyzed by electroencephalogram recordings [9]. Since caffeine mediates its effects by a blockade of $A_1$ and $A_{2A}$ adenosine receptors in the brain, these results may indicate that valerian might activate central adenosine receptors. Recently, a novel lignan derivative, an olivil glycoside, was isolated from valerian, and found to be a selective partial agonist, the first non-nucleoside agonist, at adenosine $A_1$ receptors [10]. However, further constituents may contribute to the effects of the valerian extracts at adenosine receptors.

Adenosine $A_1$ receptors are members of the P1 purine receptor family consisting of four subtypes, designated $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ [11]. The $A_1$ subtype is found in high density in many areas of the brain, including cortex, hippocampus, cerebellum, and spinal cord, and in lower density in a number of peripheral organs, including heart, kidneys, liver, skeletal muscle, adipose tissue, salivary glands, esophagus, colon, antrum, and testis [11]. Adenosine appears to be one of the main sleep-inducing substances in the brain, which accumulates during wake time, and its sedative effects may be mediated by both, adenosine $A_1$ and $A_{2A}$ receptors [12–15]. Adenosine $A_1$ receptors are coupled to inhibition of adenylate cyclase. In addition, coupling to phospholipase C, potassium channels, and calcium channels has been described [11]. Activation of adenosine $A_1$ receptors results in sedative, anticonvulsive, analgesic, antidiuretic, negative inotropic, and antiarrhythmic effects, among others [16]. Adenosine $A_1$ receptors have become important new drug targets, and $A_1$ agonists are currently being developed as antiarrhythmic and antinociceptive drugs [16], while $A_1$ antagonists are in clinical development for congestive heart failure due to their positive inotropic, kidney-protective and diuretic activities [17]. The adenosine $A_{2A}$ receptor subtype is found in high density only in specific brain areas, i.e. caudate-putamen (striatum), nucleus accumbens, and olfactory tubercle. Selective antagonists for $A_{2A}$ receptors are promising novel therapeutics for Parkinson's disease and may also exhibit neuroprotective and antidepressive activities [18]. The $A_{2B}$ and $A_3$ receptor subtypes are only expressed in low density in the brain [11].

The present study was aimed at investigating valerian extracts of different polarity and studying their interaction (binding and function) with the adenosine receptor subtypes expressed in high density in the brain, namely $A_1$ and $A_{2A}$. Our goal was to elucidate which fractions contained the highest affinity and activity, and subsequently to perform bioassay-guided fractionation in order to identify new lead structures for the development of adenosine receptor ligands.

## 2. Materials and methods

### 2.1. Instruments, chemicals and biochemicals

Infrared (IR) spectra were recorded on a Bruker TENSOR 27 IR spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained in deuterated chloroform ($CDCl_3$) as a solvent in the presence of tetramethylsilane as an internal standard on a Bruker AMX 500 NMR spectrometer. Two-dimensional NMR spectra were obtained by standard procedures as described (HH-COSY, HSQC, HMBC) [19]. Electron impact mass spectra (EIMS) were determined on a Thermoquest Finnigan MAT 95 XL mass spectrometer at 70 eV with single high resolution at the molar mass peak [$M^{+\bullet}$]. Liquid chromatography coupled to mass spectrometry (LCMS) was performed on an API 2000 (Applied Biosystems) apparatus using an electrospray ionisation (ESI) source; low pressure liquid chromatography (LPLC) was performed on Lobar® A & B material (Merck) as a solid phase. For column chromatography silica gel MN, 60 mesh (Macherey & Nagel) was used. Thin layer chromatography (TLC) was done on Merck silica $254_{GF}$ glass plates, and spots were detected by the following methods: (1) UV 254/366 nm; (2a) ammonia (vapour) treatment and subsequent detection at 366 nm; (2b) cerium(IV) sulfate spraying (0.3 g $Ce(SO_4)_2$ in 0.1 l concentrated aqueous nitric acid (65%)) followed by hot blow-drying: brown color. All chemicals and cell culture media were purchased from Sigma (Taufkirchen, Germany), unless otherwise noted.

### 2.2. Extracts

Extracts were prepared from two different commercial kinds of Polish dried valerian roots (*V. officinalis* L., Kawon (K) and Lublin (L), both trade marks), which are presumed to be rich in lignans. Cut root material was purchased in a Polish pharmacy. Extractions were performed using the finely ground roots. Yields given are refering to this material.

#### 2.2.1. Extraction method A

Powdered valerian roots (10 g) were suspended in methanol, treated in an ultrasonic bath for 5 min (four times, 20–10–10–10 ml) and filtered. Combined filtrates were concentrated followed by azeotropical drying through the addition of absolute ethanol and ether portions and subsequent evaporation (obtained extracts: hydrophilic methanol direct extracts KA-dir and LA-dir, yields: 9.9% and 11.1%, respectively). These two methanol extracts were resuspended each in 20 ml of water and first partitioned with a mixture of petroleum ether:diethyl ether (1:1, four times, 20–10–10–10 ml) to give the lipophilic petroleum ether:diethyl ether back-extracts KA-1 and LA-1 (organic layers, yields 1.4% and 1.7%, respectively). The remaining water suspensions were extracted again, three times with chloroform (giving the chloroform back-extracts KA-2 and LA-2, yields 0.4% each) and subsequently three times with *n*-butanol (yielding the butanol back-extracts KA-3 and LA-3, 1.1% and 1.9%, respectively). The remaining water suspensions were lyophilized (hydrophilic extracts KA-4 and LA-4, yields 6.9% and 8.0%, respectively). The powder residues obtained after the initial methanol extraction were further extracted with hot water (80 °C) and these extracts were lyophilized (hydrophilic extracts KA-5 and LA-5, yields 5.4% and 4.4%, respectively).

#### 2.2.2. Extraction method B

About 10 g of powdered valerian roots (K or L) were extracted four times with a mixture of petroleum ether:diethyl ether (1:1, four times, 20–10–10–10 ml) to give the lipophilic extracts KB-1 and LB-1 (yields 1.0% and 0.9%, respectively). The powder residues were subsequently extracted with four solvents ($3 \times 10$ ml solvent each), first with chloroform (chloroform extracts KB-2 and LB-2, yields 0.8% and 0.7%, respectively),

secondly with *n*-butanol (extracts KB-3 and LB-3, yields 0.6% and 0.4%, respectively), thereafter with methanol (hydrophilic extracts KB-4 and LB-4, yields 6.2% and 6.1%, respectively), and finally with hot water (80 °C) to give – after lyophilization – the hydrophilic hot water extracts KB-5 and LB-5, in yields of 12.0% and 15.2%, respectively.

#### 2.2.3. *Extraction method C*

In a third extraction variant, finely ground roots were extracted with hot water (80 °C) and the aqueous extract obtained after filtration was lyophilized (hydrophilic hot water direct extracts KC-1 and LC-1, yields 23.6% and 27.3%, respectively).

### 2.3. *Isolation of isovaltrate (1, KL-B-1$_{MeOH}$-III-D-b)*

Powdered valerian roots (1700 g, a mixture of Kawon (K) and Lublin (L), 1:1) were mazerated three times overnight with 4 l portions of petroleum ether (40–60 °C):diethyl ether (1:1) in a rotating flask. After evaporation, the resulting viscous oil (35.4 g lipophilic extract KL-B-1) was dissolved in 70 ml of methanol and lipids were allowed to precipitate by leaving the solution at 4 °C for 24 h. The supernatant liquid was removed and subsequently evaporated yielding 11.8 g of a methanol-soluble fraction (KL-B-1$_{MeOH}$). A part (10 g) thereof was resuspended in 22 ml methanol, and 7 g of solid Sephadex LH20 material was added upon stirring. After soaking overnight, the suspension was poured onto a Sephadex column (1 kg, equilibrated with MeOH) followed by elution with MeOH. By TLC monitoring (*n*-hexane:ethyl acetate, 7:3), eight subfractions were collected. Subfraction KL-B-1$_{MeOH}$-III yielded 4.33 g of material; a portion (1.0 g) was rechromatographed on a Lobar B Si-60 column using *n*-hexane:methyl acetate:ethyl acetate = 84:8:8 as an eluent, and fractions containing a compound with an $R_f$ value of ~0.7 were combined yielding 175 mg of KL-B-1$_{MeOH}$-III-D as a crude oil. KL-B-1$_{MeOH}$-III-D was further purified on silica gel (Lobar A Si-60 column, chloroform:ethyl acetate = 9:1) yielding 85 mg ($R_f$ 0.57) of a faintly yellow oil (KL-B-1$_{MeOH}$-III-D-b). Analytical data showed that this fraction consisted of isovaltrate [20] (≥95% purity) containing an impurity (ca. 3%) of homoisovaltrate.

### 2.4. *Analytical data for the isolated isovaltrate (butanoic acid 3-methyl-(1S,2′R,6S,7aS)-6-(acetyloxy)-6,7a-dihydro-4-[(3-methyl-1-oxobutoxy)methyl]spiro[cyclopenta[c]pyran-7(1H),2′-oxiran]-1-yl ester)*

Isovaltrate (**1**) [21,22] was obtained as a faintly yellowish oil; its infrared and mass spectra were in accordance with literature data [22,23]. Standard NMR experiments (HH-COSY, HSQC, HMBC [19]) were performed to correlate all $^1$H and $^{13}$C shift values. The signal multiplicities are cited as: brs, broad singlet; d, doublet; dd, doubled doublet; t, triplet; sept, septet. Coupling constants are given in Hz.

$^1$H NMR: 5.94 d (10.0 Hz, 1-H); 6.67 brs (3-H); 5.82 t (~2.7 Hz, 6-H); 5.34 d (2.7 Hz, 7-H); 3.39 dd (10.0/2.5 Hz, 9-H); 2.21 dd (14.3/~7 Hz, 2′-H$_A$); 2.18 dd (14.3/~7 Hz, 2′-H$_B$); 2.08 2× sept overlapping (6.8 Hz, 3′-H and 3″-H); 0.96 d (6H, 6.7 Hz, 2× Me at C3′); 4.73 and 4.63 d (CH$_2$ at C-4, $J_{A,B}$ = 12.3 Hz); 2.18 dd overlapping (~14/~6.5 Hz, 2″-H$_A$); 2.15 dd overlapping (~14/~6.5 Hz, 2″-H$_B$); 0.92 d (6H, 6.7 Hz, 2× Me at C3″); 2.02 s (3H, 7-OAc-Me); 2.87 d, 3.01 d (oxirane, 10-CH$_2$, $J_{A,B}$ 12.3 Hz).

$^{13}$C NMR: 92.5 (C-1); 148.4(C-3); 108.3 (C-4); 140.9 (C-5); 118.5 (C-6); 83.2 (C-7); 64.1 (C-8); 42.6 (C-9); 60.6 (CH$_2$ at C-4); 47.8 (oxirane, 10-CH$_2$); 170.3 (2C, C-1′ and C=O acetyl); 42.9 (C-2′); 25.5 (2C, C-3′, C-3″); 22.4 (4× CH$_3$ at C-3′, C-3″); 172.9 (C-1″); 43.1 (C-2″); 21.0 (CH$_3$ acetyl).

### 2.5. *Radioligand binding assays*

Frozen rat brains were obtained from Pel Freez®, Rogers, Arkansas, USA, and thawed at 4 °C. Frontal cortex was dissected as $A_1$ adenosine receptor source. Right and left striata were dissected for $A_{2A}$ adenosine receptor binding 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 [24,25]. Final protein pellets were resuspended in 50 mM Tris–HCl buffer pH 7.4 and stored in aliquots at −80 °C until use. The protein concentration was determined by the method of Bradford, using a BIOrad assay kit. Before determination, the protein was washed in HEPES-NaOH buffer (10 mM, pH 7.4) to prevent interactions of the Tris–HCl buffer with the reagents used for colorimetric analysis of the protein concentration. Bovine serum albumin was used as a reference standard. Membranes were preincubated with 0.2 IU/ml of adenosine deaminase (ADA) in order to remove endogenous adenosine. The extracts were dissolved in DMSO, and 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.

The valerian extracts were investigated in radioligand binding assays at adenosine $A_1$ receptors of rat brain cortical membranes using the $A_1$-selective radioligand [$^3$H]2-chloro-N$^6$-cyclopentyladenosine ([$^3$H]CCPA, 1576 TBq/mmol, Perkin-Elmer, Germany), and at $A_{2A}$ adenosine receptors of striatal membranes using the $A_{2A}$-selective radioligand [$^3$H]3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargyl-xanthine ([$^3$H]MSX-2, 3108 TBq/mmol, custom-labeled by Amersham, Germany). Radioligand binding assays were carried out in Tris–HCl buffer 50 mM, pH 7.4, as previously described [26,27]. $A_1$ assays were incubated at room temperature for 90 min. Nonspecific binding was defined using 10 μM of 2-chloroadenosine and amounted to less than 5% of total binding. [$^3$H]CCPA was used in a final concentration of 1 nM. Protein (ca. 30 μg per well containing a final volume of 200 μl) was added to start the reaction. Incubation was terminated by rapid filtration using a Brandel 96-channel cell harvester (Brandel, Gaithersburgh, Maryland, USA) through GF/B glass fiber filter plates (Perkin-Elmer, Boston, USA). Filters were rinsed three times with ice-cold Tris–HCl buffer 50 mM, pH 7.4. Radioactivity of the wet filter plates was counted after 9 h of preincubation with 50 μl of Microscint 20 scintillation cocktail (Perkin-Elmer, Boston, USA).

$A_{2A}$ assays were incubated at room temperature 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. [$^3$H]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. The incubation was terminated by rapid filtration through GF/B glass fiber filters (Schleicher and Schuell, Germany), presoaked in 0.5% aqueous polyethylenimine solution for 30 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 2.5 ml of Ultima Gold scintillation cocktail (Perkin-Elmer, Boston, USA).

### 2.6. [$^{35}$S]GTPγS assays

Membrane preparations of CHO cells recombinantly expressing the human adenosine $A_1$ receptor (CHO-h$A_1$ cells) (5 μg per tube) were incubated with 0.5 nM [$^{35}$S]GTPγS (46.3 TBq/mmol, Perkin-Elmer) in a total volume of 200 μl in 50 mM Tris–HCl buffer pH 7.4 containing 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 μM GDP, 100 mM NaCl, 2 IU/ml adenosine deaminase (ADA), 0.5% bovine serum albumin, and extract or test compound, according to Lorenzen et al. [28]. Nonspecific binding was determined with 10 μM of unlabeled GTPγS. Incubations were terminated after 45 min at room temperature by the addition of 2 ml of ice-cold buffer containing 50 mM Tris–HCl pH 7.4 and 5 mM $MgCl_2$, and rapid filtration through GF/B glass fiber filters (Schleicher and Schuell, Germany) on a Brandel 48-channel cell harvester, followed by two washing steps with ice-cold buffer. Radioactivity on the filters was measured by liquid scintillation counting after punching out the filters and filling up the vials with 2.5 ml of Ultima gold scintillation cocktail (Perkin-Elmer, Boston, USA).

### 2.7. Cell culture

CHO cells stably transfected with the human $A_1$ adenosine receptor were grown adherently and maintained in Dulbecco's modified Eagles medium F12, supplemented with 10% fetal calf serum, penicillin (100 IU/ml), streptomycin (100 μg/ml) and L-glutamate (2 mM) at 37 °C, 5% $CO_2$. 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 in phosphate buffered saline and kept frozen at −80 °C until membrane preparation following the procedure described by Klotz et al. [29].

### 2.8. Preparation of brain slices

Cortical slices of the rat brain were prepared and maintained as previously described [30,31]. In brief, male Wistar rats (own breed; 150–200 g) were anesthetized with diethyl ether and decapitated. The brain was rapidly removed and submerged in cold (−1 °C) artificial cerebrospinal fluid (ACSF) saturated with 95% $O_2$–5% $CO_2$. The ACSF contained (mM): NaCl, 126; KCl, 2.5; $NaH_2PO_4$, 1.2; $MgCl_2$, 1.3; $CaCl_2$, 2.4; $NaHCO_3$, 25; glucose, 11; ascorbic acid, 0.3; ethylenediamine tetraacetic acid, 0.03; pH 7.4. Five coronal slices (300 μm thick) were cut with a vibratome (TSE Systems GmbH, Bad Homburg, Germany) from a block of the rat brain including the cingulate cortex. The slices were initially transferred to an interface holding chamber maintained at room temperature in 250 ml oxygenated ACSF in order to equilibrate. An individual slice was transferred to a recording chamber, where it was placed on a nylon net and superfused continuously with oxygenated ACSF (34 °C) at a rate of 2 ml/min. Electrophysiological recordings were started at least one hour after slice preparation.

### 2.9. Identification of pyramidal cells and intracellular recordings

Intracellular recordings were obtained from pyramidal cells of the cingulate cortex in layer V. All neurons were electrophysiologically identified as regular-spiking cells by injection of depolarizing current pulses (0.4–0.6 nA, 95 ms, 2.5 Hz). Only one cell was used for electrophysiological recording from each slice.

Recordings of the membrane potential and current injection were carried out with glass microelectrodes filled with 2 M KCl (tip resistance 50–120 MΩ) using a high impedance preamplifier and a bridge circuit (Axoclamp-2B; Axon Instruments, Foster City, CA, USA). In all experiments, pyramidal cells were hyperpolarized by approximately 10–15 mV by injecting hyperpolarizing current to prevent spike activity during the experiment. The resting membrane potential was determined by withdrawal of the microelectrode from the cell at the end of each experiment.

### 2.10. Electrical stimulation and application of drugs

PSPs were evoked by electrical stimulation (0.2 Hz, 1–2 ms, 20–120 V) with a concentric bipolar tungsten electrode (World Precision Instruments Inc., Sarasota, FL, USA) placed in layer I. The stimulation voltage was adjusted individually for each slice to yield PSP amplitudes, which were approximately 80% of maximum. The drugs or the valerian extracts were applied by changing the superfusion medium by means of three-way valves. The effects on the PSPs were analysed by continuous superfusion for 5 min. The reversibility of the effects was tested by washout (10–30 min).

### 2.11. Data analysis

Data were analyzed using GRAPHPAD PRISM Version 4.0 (San Diego, CA, USA). For non-linear regression analysis, the Cheng Prusoff equation and $K_D$ values of 0.2 nM for [$^3$H]CCPA and 8 nM for [$^3$H]MSX-2 were used to calculate $K_i$ values from $IC_{50}$ values. GTPγS binding 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.

The electrophysiological data are expressed as means ± S.E.M. from $n = 3$ independent experiments. Multiple comparisons were performed by one-way analysis of variance followed by Bonferroni's *t*-test. The comparisons between groups were made by the paired Student's *t*-test. A probability level of 0.05 or less was considered to be statistically significant.

## 3. Results

Two different commercial samples of roots of *V. officinalis* L. obtained from Poland, named "Kawon" (K) and "Lublin" (L),

were subjected to extraction procedures to obtain a series of extracts of different polarity, from very polar to highly nonpolar. Three extraction methods were applied (see Fig. 1): (i) methanol extraction and, after drying, step-wise back-extraction of the methanolic extract with solvents of decreasing lipophilicity; (ii) extraction with a highly lipophilic solvent mixture (petroleum ether:diethyl ether = 1:1) and, after drying, subsequent extraction in several steps with solvents of increasing polarity; (iii) extraction with hot water (see Fig. 1).

The obtained valerian extracts were investigated in radioligand binding assays for their affinity to adenosine $A_1$ and $A_{2A}$ receptors. Rat brain cortical membranes and the radioligand [$^3$H]N$^6$-cyclopentyladenosine ([$^3$H]CCPA) were used for the $A_1$ receptor assays, rat brain striatal membranes and [$^3$H]3,7-dimethyl-1-propargyl-8-(*m*-methoxystyryl)xanthine ([$^3$H]MSX-2) were used for $A_{2A}$ assays. The results obtained for all raw extracts tested at a concentration of 100 μg/ml are listed in Table 1. In most cases, Lublin and Kawon extracts gave very similar results. Clear exceptions appeared to be KA-2 and LA-2, two lipophilic back-extracts of the methanolic extract, since LA-2 showed significantly higher adenosine receptor affinity as compared to KA-2.

All of the extracts showed no or only low to moderate affinity for adenosine $A_{2A}$ receptors, usually inhibiting radioligand binding by less than 35% at a test concentration of 100 μg of extract per ml. There was one exception: LA-2, the chloroform back-extract of the methanolic extract (extraction method A, see Fig. 1) showing 57% inhibition of radioligand binding at a concentration of 100 μg/ml. It appears that lipophilic constituents are present in valerian that exhibit a certain affinity for the adenosine $A_{2A}$ receptor subtype (e.g. KA-1 and LA-1, LA-2, KB-1 and LB-1). However, the number of extracts which exhibited affinity for adenosine $A_1$ receptors was much higher. Thus, we focussed on extracts with high affinity for $A_1$ receptors for further characterization and to perform bioassay-guided fractionation. High binding affinity was found in lipophilic as well as in more hydrophilic fractions. For selected extracts inhibition curves were determined in order to obtain $K_i$ values. The lipophilic extracts KB-1 and LB-1, KB-2 and LB-2, and KB-3 as well as the extract LA-2 and the methanolic extract LA-dir were investigated. The lipophilic extracts exhibited $K_i$ values ranging from 7.16 to 516 μg/ml, while the methanolic extract showed a $K_i$ value of 139 μg/ml and its chloroform back extract LA-2 a $K_i$ value of 39 μg/ml.

In order to characterize the kind of interaction – agonistic or antagonistic – [$^{35}$S]GTPγS binding studies were performed. Such studies were performed for five of the most potent extracts of different polarity, LA-2, KB-1, KB-2, KB-3, and LA-dir. LB-2 was excluded due to its low affinity, and LB-1 was not investigated since it showed nearly the same affinity as KB-2, an extract of the same polarity. For these studies, recombinant human $A_1$ receptors expressed in high density in Chinese hamster ovary (CHO) cells were used. Receptor ligands can allosterically modulate the binding of the metabolically stable, radiolabelled GTP analog [$^{35}$S]GTPγS to the G protein. While agonists will increase the binding of [$^{35}$S]GTPγS, neutral antagonists would not have any effect, and antagonists with inverse agonistic activity will lead to a decrease in [$^{35}$S]GTPγS binding [32]. The use of a cell line with high receptor expression level allows the monitoring of inverse agonists. [$^{35}$S]GTPγS binding can also be done in native tissues like rat cortical membranes [7], but the effects of inverse agonists are

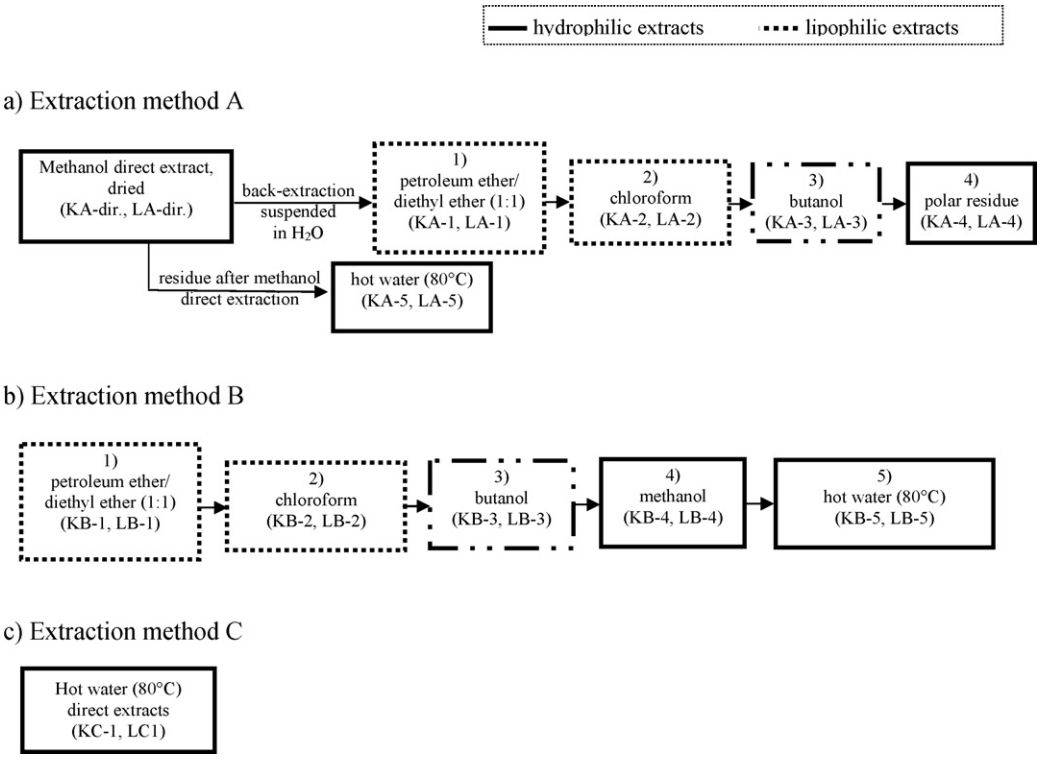


**Fig. 1 – Extraction methods for valerian extracts (K = "Kawon", L = "Lublin" product).**

**Table 1 – Affinities of valerian raw extracts at rat adenosine $A_1$ and $A_{2A}$ receptors**

| Extract | $A_1$ vs. [$^3$H]CCPA at rat brain cortical membranes | | $A_{2A}$ vs. [$^3$H]MSX-2 at rat brain striatal membranes | |
|---|---|---|---|---|
| | Percent inhibition of radioligand binding at 100 μg/ml ($n$ = 2)[a] | $K_i$ ± S.E.M. (μg/ml) ($n$ = 3)[a] | Percent inhibition of radioligand binding at 100 μg/ml ($n$ = 2)[a] | $K_i$ ± S.E.M. [μg/ml] ($n$ = 3)[a] |
| KA-1 | 40 ± 13 | n.d. | 33 ± 2 | n.d. |
| LA-1 | 18 ± 14 | n.d. | 34 ± 2 | n.d. |
| KA-2 | 8 ± 10 | n.d. | 20 ± 5 | n.d. |
| LA-2 | 50 ± 10 | 39.0 ± 4.0 | 57 ± 4 | n.d. |
| KA-3 | 12 ± 11 | n.d. | 11 ± 4 | n.d. |
| LA-3 | 25 ± 11 | n.d. | 11 ± 2 | n.d. |
| KA-4 | 4 ± 3 | n.d. | 4 ± 2 | n.d. |
| LA-4 | 25 ± 10 | n.d. | 15 ± 7 | n.d. |
| KA-5 | 13 ± 10 | n.d. | 10 ± 3 | n.d. |
| LA-5 | 19 ± 10 | n.d. | 4 ± 4 | n.d. |
| KB-1 | 56 ± 8 | 80.8 ± 16.0 | 25 ± 3 | n.d. |
| LB-1 | 47 ± 8 | 53.0 ± 10.0 | 32 ± 4 | n.d. |
| KB-2 | 56 ± 5 | 7.16 ± 0.02 ($n$ = 2) | 21 ± 2 | n.d. |
| LB-2 | 46 ± 3 | 516 ± 43 | 17 ± 10 | n.d. |
| KB-3 | 48 ± 6 | 88.5 ± 19.0 | −2 ± 3 | n.d. |
| LB-3 | 30 ± 5 | n.d. | −3 ± 6 | n.d. |
| KB-4 | 5 ± 13 | n.d. | −11 ± 2 | n.d. |
| LB-4 | 19 ± 13 | n.d. | −7 ± 5 | n.d. |
| KB-5 | 5 ± 13 | n.d. | −14 ± 3 | n.d. |
| LB-5 | 21 ± 13 | n.d. | −14 ± 3 | n.d. |
| KA-dir | 16 ± 2 | n.d. | 2 ± 7 | n.d. |
| LA-dir | 21 ± 5 | 139 ± 18 | 0 ± 0 | n.d. |
| KC-1 | 12 ± 5 | n.d. | 2 ± 8 | n.d. |
| LC-1 | 22 ± 6 | n.d. | −6 ± 5 | n.d. |

[a] Unless otherwise noted.

usually too small to be monitored, and only agonistic and antagonistic activity can be distinguished. As reference compounds, the full $A_1$ agonist $N^6$-cyclopentyladenosine (CPA) and the most efficacious inverse agonist known to date, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were investigated in the same assay. Obtained data were normalized with respect to the maximal effect of CPA set at 100% (Fig. 2 and Table 2). CPA led to a concentration-dependent enhancement in [$^{35}$S]GTPγS binding (maximal effect: 236 ± 25%, $n$ = 5) with an $EC_{50}$ value of 0.825 nM. DPCPX showed an inhibition of [$^{35}$S]GTPγS binding by 40% of the basal level with an $EC_{50}$ value of 2.11 nM. While the lipophilic extract KB-1 showed a strong inhibition of [$^{35}$S]GTPγS binding, the hydrophilic extract LA-dir stimulated [$^{35}$S]GTPγS binding by 49% with respect to CPA indicating that the lipophilic extract was inverse agonistic while the hydrophilic extract showed partial agonistic activity. The extracts of intermediate polarity (LA-2, KB-3) exhibited a much lower degree of stimulation of [$^{35}$S]GTPγS binding (21–26%) than the methanolic extract LA-dir (49% stimulation with respect to CPA). KB-2, a relatively lipophilic extract, showed nearly no effect on [$^{35}$S]GTPγS binding, although it had high affinity for the receptor. Table 3 shows that the $EC_{50}$ values obtained in [$^{35}$S]GTPγS assays at human $A_1$ receptors were consonant with the $K_i$ values from binding studies at rat $A_1$ receptors.

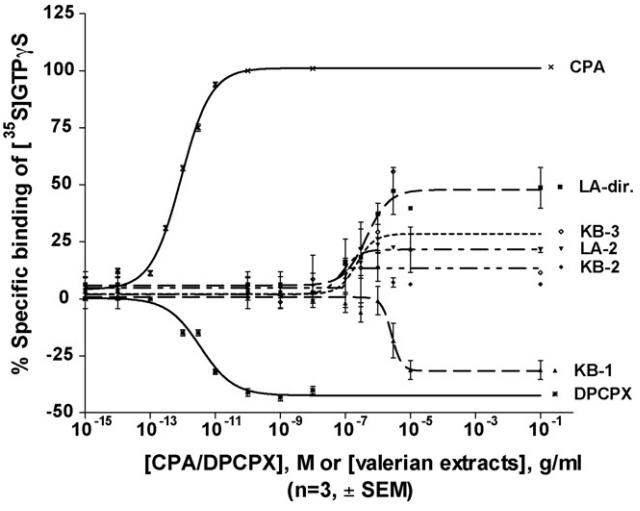


**Fig. 2 – [$^{35}$S]GTPγS binding study at recombinant human $A_1$ adenosine receptors, normalized data with respect to CPA as a full agonist set as 100% maximal stimulation.**

As can be seen in Fig. 3, the agonistic effects of the polar valerian extracts (e.g. LA-dir, LA-2, KB-3) could be completely blocked by the addition of a high concentration of the $A_1$-selective inverse agonist DPCPX. Similarly, the inverse agonistic effect of the lipophilic extract KB-1 was completely blocked by the addition of a high concentration of the $A_1$-selective agonist CPA. These experiments proved that the effects of the extracts on [$^{35}$S]GTPγS binding were indeed mediated via adenosine $A_1$ receptors in the recombinant CHO cells expressing a high density of the human receptor.

In order to identify the active principle of the lipophilic extract with inverse agonistic activity we subjected it to further fractionation. Since both products, ''Kawon'' and ''Lublin'' had shown nearly identical receptor affinity (Table 1), a mixture of both (named KL-B-1) was used for

**Table 2 – $K_i$-values at rat adenosine $A_1$ receptors, $EC_{50}$-values at recombinant human $A_1$ receptors and percent maximal stimulation in relation to the maximal effect of the full agonist CPA (=100%) of selected valerian raw extracts**

| Extract/compound | $K_i \pm$ S.E.M. at adenosine $A_1$ receptor (rat) (μg/ml) ($n = 3$) | GTPγS binding $EC_{50} \pm$ S.E.M. (μg/ml) ($n = 3$) (human recombinant $A_1$ receptor) | % maximum stimulation related to maximum effect of the full agonist CPA (=100%) ($n = 3$) |
|---|---|---|---|
| CPA (full agonist) | 0.32 nM | 0.852 ± 0.611 nM | 100 |
| DPCPX (full inverse agonist) | 0.90 nM | 2.11 ± 0.24 nM | −40 ± 2 |
| LA-dir (methanol direct extract) | 139 ± 18 | 489 ± 202 | 49 ± 9 |
| LA-2 (chloroform) | 39.0 ± 4.0 | 60 ± 36 | 21 ± 1 |
| KB-1 (petrol ether/ diethyl ether, 1:1) | 80.8 ± 16.0 | 319 ± 55 | −31 ± 4 |
| KB-2 (chloroform) | 7.16 ± 0.02 | 119 ± 108 | 8 ± 2 |
| KB-3 (butanol) | 88.5 ± 19.0 | 453 ± 431 | 26 ± 2 |

**Table 3 – $K_i$-values at rat adenosine $A_1$ receptors, $EC_{50}$-values at recombinant human $A_1$ receptors and percent maximal stimulation in relation to the maximal effect of the full agonist CPA (=100%) of the valerian extract fractions KL-B1-I–VIII**

| K/L-B1 fraction | $K_i \pm$ S.E.M. at adenosine $A_1$ receptor (rat) (μg/ml) ($n = 3$) | GTPγS binding $EC_{50} \pm$ S.E.M. (μg/ml) ($n = 3$) (human recombinant $A_1$ receptor) | % maximum stimulation related to maximum effect of the full agonist CPA (=100%) ($n = 3$) |
|---|---|---|---|
| I | 194 ± 106 | n.d. | n.d. |
| II | 26.0 ± 4.0 | n.d. | n.d. |
| III | 9.83 ± 0.48 | 11.4 ± 3.4 | −46 |
| IV | 29.1 ± 2.2 | n.d. | n.d. |
| V | 9.65 ± 3.86 | 272 ± 84 | −23 |
| VI | 6.96 ± 0.15 | 50.0 ± 4.3 | −23 |
| VII | 10.0 ± 0.8 | 106 ± 4 | −37 |
| VIII | 30.8 ± 6.8 | n.d. | n.d. |

isolation of active constituents. The petroleum ether–diethyl ether extract (KL-B-1) was submitted to a Sephadex LH-20 column for separation according to molecular size. After elution with methanol, eight crude fractions (KL-B-1-I–VIII) of different volumes were collected according to their TLC behaviour (UV detection at 254 nm and spraying with cerium(IV) sulfate). $A_1$ affinities were determined at rat brain receptors, and subsequently, the most potent fractions III, V, VI, and VII ($K_i$ values 9.65–10.0 μg/ml) were additionally investigated in [$^{35}$S]GTPγS assays at human receptors for their functional properties (Table 3). Indeed, all four fractions exhibited inverse agonistic activity, although with different efficacies. The most efficacious fraction was KL-B-1-III, which behaved as a full inverse agonist (46% maximal inhibition) in comparison to DPCPX (40% maximal inhibition). Fractions V–VII inhibited [$^{35}$S]GTPγS binding by 23% (V, VI), and 37% (VII), respectively.

Fraction III exhibiting the highest degree of inverse agonistic activity was further subfractionated by column chromatography on silica gel, and eight subfractions (KL-B-1-III-A–H) were obtained. All of the subfractions were investigated in radioligand binding studies at rat adenosine $A_1$ as well as $A_{2A}$ receptors (Table 4). Tested at a concentration of 100 μg/ml fraction D showed the highest affinity to both receptor subtypes, while all other fractions showed less than 50% inhibition of radioligand binding. This most potent fraction was further purified by another silica gel column chromatography step yielding two final fractions, a and b, which were again investigated in radioligand binding studies at rat $A_1$ receptors and for functional properties in [$^{35}$S]GTPγS studies at human $A_1$ receptors. The results are shown in Table 5 and Fig. 4. The highest receptor affinity was detected in the second fraction (KL-B-1-III-D-b), which was finally purified by a third silica gel column chromatography to yield product **1**. The compound (**1**) showed potent inverse agonistic activity being about as potent as DPCPX, the most potent inverse agonist at $A_1$ receptors known to date, and exhibited high affinity for $A_1$ receptors ($K_i$ 0.865 μg/ml, 2.05 μM); its $EC_{50}$ value obtained

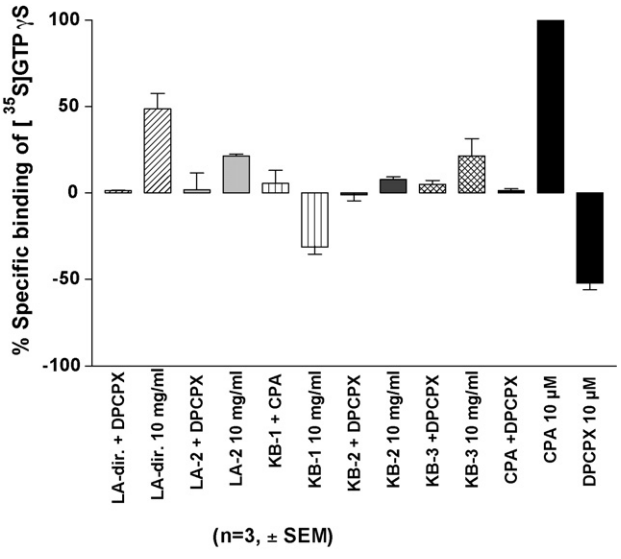


**Fig. 3 – [$^{35}$S]GTPγS binding study at recombinant human adenosine $A_1$ receptors in the presence/absence of a high concentration (100 nM) of DPCPX ($A_1$ antagonist) or CPA ($A_1$ agonist).**

**Table 4 – Affinity of valerian extract fractions KL-B1-III-A–H at rat adenosine $A_1$ and $A_{2A}$ receptors**

| K/L-B1-III fraction | $A_1$ vs. [$^3$H]CCPA rat brain cortical membranes (percent inhibition of radioligand binding at 100 μg/ml ($n$ = 3)) | $A_{2A}$ vs. [$^3$H]MSX-2 rat brain striatal membranes (percent inhibition of radioligand binding at 100 μg/ml ($n$ = 3)) |
|---|---|---|
| Mixture (A–H) | 61 ± 2 | 20 ± 3 |
| A | −4 ± 4 | 19 ± 6 |
| B | 18 ± 7 | 9 ± 1 |
| C | 17 ± 13 | 20 ± 7 |
| D | 70 ± 8 | 51 ± 0 |
| E | 51 ± 2 | 15 ± 9 |
| F | 38 ± 4 | 30 ± 4 |
| G | 20 ± 1 | 23 ± 3 |
| H | 24 ± 4 | 21 ± 8 |

**Table 5 – $K_i$ values at rat adenosine $A_1$ receptors and $EC_{50}$-values at recombinant human $A_1$ receptors of two subfractions of the KL-B1 fraction**

| K/L-B1 fraction | $A_1$ vs. [$^3$H]CCPA at rat brain cortical membranes | | [$^{35}$S]GTPγS assay at CHO cell membranes recombinantly expressing the human $A_1$ receptor | |
|---|---|---|---|---|
| | Percent inhibition of radioligand binding at 100 μg/ml ($n$ = 2) | $K_i$ ± S.E.M. (μg/ml) ($n$ = 3) | $EC_{50}$ ± S.E.M. (μg/ml) ($n$ = 3) | % maximum stimulation related to maximum effect of the full agonist CPA (=100%) ($n$ = 3) |
| III-D-a | 9 ± 7 | n.d. | n.d. | n.d. |
| III-D-b (impure fraction) | 70 ± 2 | 1.29 ± 0.18 | n.d. | n.d. |
| Purified fraction III-D-b (isovaltrate) | 71 ± 2 | 0.865 ± 0.131 μg/ml = 2.05 ± 0.31 μM | 1.94 ± 0.40 | −56 ± 2 |
| Caffeine | n.d. | 44 μM | n.d. | n.d. |

from GTPγS binding studies (1.94 μg/ml) correlated well with the $K_i$ value. The structure of compound **1** was elucidated by MS and 2D NMR experiments (COSY; HSQC, HMBC) [19]. A comparison with published data revealed that **1** was isovaltrate (see Fig. 5), a well known lipophilic constituent of valerian which had first been mentioned by Thies and Funke in 1967 [33].

Isovaltrate (**1**) exhibited a $K_i$ value of 2.05 μM for rat $A_1$ receptors (Table 5). In order to further study the pharmacological properties of isovaltrate (**1**) we investigated its

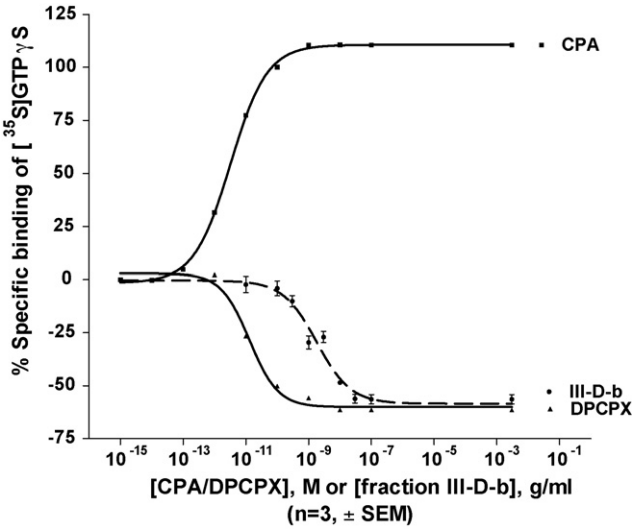


**Fig. 4 – [$^{35}$S]GTPγS binding at recombinant human adenosine $A_1$ receptors of the fractionated extract KL-B-1-III-D-b, which was identified as isovaltrate, a lipophilic constituent of valerian.**

effects on the post-synaptic potentials in rat cortical neurons. The pyramidal cells of the cingulate cortex included in this study had a resting membrane potential of −96.5 mV and an input resistance of 57.6 MΩ. All neurons fired repetitive action potentials by injection of depolarizing current pulses. The selective $A_1$ agonist CPA (10 μM) inhibited the PSP amplitude by 51 ± 3% (Fig. 6). The effect was reversed within 30 min of washout. Superfusion of the brain slices with isovaltrate (20 μM) alone had no apparent effect on the PSPs. However, CPA (10 μM) co-applied with isovaltrate (20 μM) resulted in an inhibition of 24 ± 5% indicating a reduced inhibition of the PSPs by CPA in the presence of isovaltrate (Fig. 6).

## 4. Discussion

In previous studies it had been found that hydrophilic extracts of valerian bound to adenosine $A_1$ receptors [7] and [$^{35}$S]GTPγS assays as well as adenylate cyclase assays had shown that hydrophilic valerian extracts exhibited (partial) agonistic activity at rat and human adenosine $A_1$ receptors [7]. We had identified a new lignan glycoside, 4′-*O*-β-D-glycosyl-9-*O*-(6″-deoxysaccharosyl)olivil, which was one of the constituents that contributed to this effect [10]. In the present study we investigated valerian extracts of a wide range of different polarities, from highly polar to highly lipophilic. Our results indicated that different constituents of valerian, lipophilic as well as hydrophilic ones, interact with adenosine $A_1$ receptors in rat brain cortical membrane preparations. Most of the extracts were highly selective for rat brain cortical $A_1$ versus rat brain striatal $A_{2A}$ receptors. In fact, we could show for the

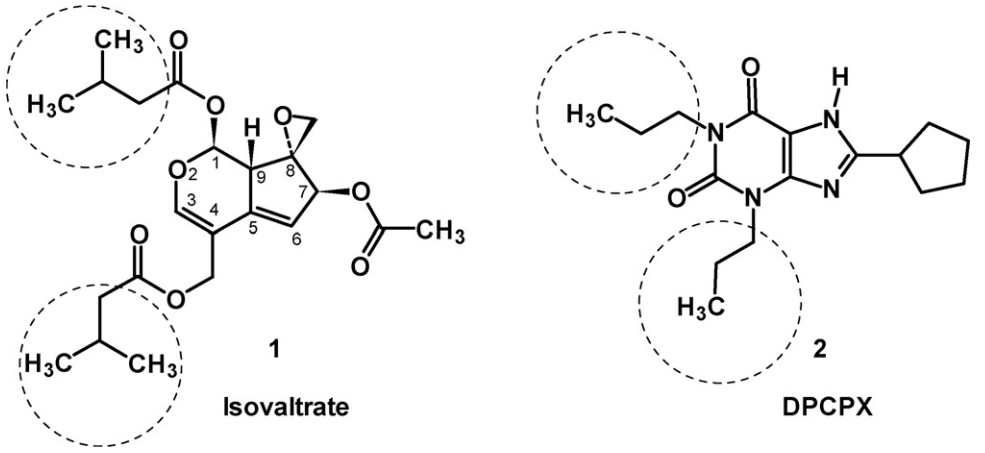


**Fig. 5 – Structural comparison of the adenosine $A_1$ receptor inverse agonists isovaltrate (*1*) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 2). Both molecules contain two lipophilic domains occupied by alkyl groups (circles).**

first time that not only hydrophilic, but also lipophilic extracts of valerian exhibited affinity for adenosine $A_1$ receptors. But while polar extracts were partial agonistic, highly lipophilic extracts (KB-1) were inverse agonistic as shown by [$^{35}$S]GTPγS binding to membrane preparations of CHO cells recombinantly expressing the human $A_1$ receptor. The effects of valerian extracts on [$^{35}$S]GTPγS binding could be blocked by $A_1$-selective standard ligands. These results indicate that the observed effects are actually due to allosteric modulation of GTPγS binding to $G_i$ protein by constituents of valerian (agonists, inverse agonists) that are binding to the adenosine $A_1$ receptor. While the $EC_{50}$ values obtained in GTPγS binding studies for some extracts (e.g. LA-dir, LA-2, K/L-B1-III, Tables 2 and 3) showed an excellent correlation with the respective $K_i$ values determined in binding studies, the $EC_{50}$ values of the less efficacious fractions (e.g. KB-2, K/L-B1-V–VII) correlated less well with their $K_i$ values. The reason for this could be that the latter fractions contained mixtures of inverse agonistic, neutral antagonistic, and/or agonistic compounds, which would result in a reduced efficacy and a right-shift (higher $EC_{50}$-values) of the dose-response curves. Species differences between rat and man might also contribute to the observed discrepancies since $K_i$ values were determined in rat brain cortical membrane preparations, while GTPγS binding was performed at human receptors expressed in CHO cells.

Bioassay-guided fractionation of the lipophilic extracts finally led to the isolation of a single compound (fraction III-D-b, Table 5) whose structure could be elucidated by spectroscopic methods. It was identified as isovaltrate (**1**), a known constituent of valerian. Isovaltrate (**1**) exhibited a $K_i$ value of 2.05 μM for rat $A_1$ receptors and is thus 21-fold more potent than the standard antagonist caffeine ($K_i$ 44 μM [34]), another natural product, which is a non-selective adenosine receptor antagonist. In contrast to caffeine, which does not exhibit high efficacy as an inverse agonist, isovaltrate is a full inverse agonist with similar efficacy as DPCPX (Fig. 4). A comparison of the structures of isovaltrate (**1**) and DPCPX (**2**) (Fig. 5) shows similarities. Both compounds contain a 6:5-bicyclic core

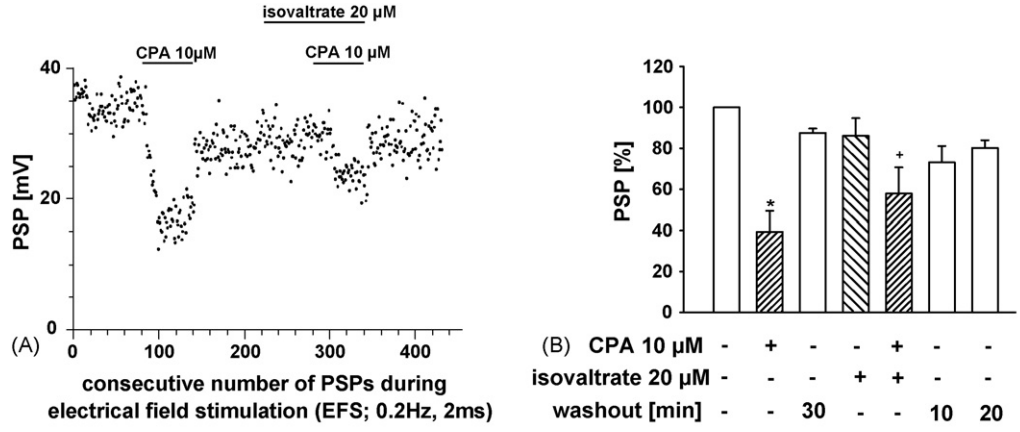


**Fig. 6 – Effects of CPA alone, and CPA in the presence of isovaltrate on PSPs evoked by electrical field stimulation in pyramidal cells of the rat cingulate cortex. (A) Representative time course of the PSPs. (B) Statistical evaluation of the inhibitory effect of CPA (10 μM) alone, and co-application of CPA (10 μM) and isovaltrate (20 μM). The effect by CPA was statistically significant (compare control and CPA effect, $^{*}P < 0.05$), the reversal of the CPA effect in the presence of isovaltrate was also statistically significant (compare effect of CPA alone and in the presence of isovaltrate, $^{+}P < 0.05$).**

structure, in the case of DPCPX a nearly flat purine ring system, in the case of isovaltrate a partly hydrogenated cyclopenta[c]-pyran structure, which is, however, not completely flat. Both compounds have two alkyl chains (propyl in DPCPX, isobutyl as part of the isopentanoyl esters in isovaltrate). Hydrogen bond acceptors are found in both molecules, e.g. C2- and C6-carbonyl in DPCPX, ester groups in isovaltrate (Fig. 5).

Isovaltrate was further investigated in electrophysiological studies at rat brain slices. The adenosine $A_1$ receptor agonist CPA inhibited the amplitude of post-synaptic potentials in the cortical neurons (Fig. 6). In the applied experimental setting rather high concentrations of receptor ligands (e.g. 10 μM of CPA) have to be applied. Isovaltrate, applied at a concentration of 20 μM, did not completely reverse the effect of 10 μM CPA. However, the limited water-solubility precluded the application of higher concentrations of the lipophilic isovaltrate. Our experiments clearly showed that isovaltrate can antagonize the effects of an $A_1$-selective adenosine receptor agonist in rat brain slices. This is an indication that isovaltrate may exhibit stimulatory effects on the central nervous system by blockade of tonically activated adenosine $A_1$ receptors in the brain.

## 5. Conclusions

Valerian extracts of different polarities were investigated for their affinities to adenosine $A_1$ and $A_{2A}$ receptors. Polar as well as nonpolar extracts were found to interact with adenosine $A_1$ receptors. While polar extracts activated $A_1$ receptors, nonpolar extracts were antagonists or even inverse agonists at $A_1$ receptors. Isovaltrate, a lipophilic constituent of valerian, was identified as a potent and highly efficacious inverse agonist. In experiments at rat brain slices measuring PSPs in cortical neurons, isovaltrate (partly) reversed the reduction in the PSPs induced by the adenosine $A_1$ receptor agonist CPA. Thus, valerian contains lipophilic constituents, such as isovaltrate, that may counteract the desired sedative activities, and it therefore makes sense to use exclusively hydrophilic extracts for sleep-inducing, sedating therapy. However, in vivo studies will be required to evaluate the pharmacological relevance of the effects observed in vitro. Isovaltrate can be used as a new lead structure for the development of inverse agonists for adenosine $A_1$ receptors.

## Acknowledgments

We are grateful for support by the Deutsche Forschungsgemeinschaft (DFG), Graduiertenkolleg GRK-677. Thanks are due to Prof. Jerzy Palka, PhD, Medical University of Bialystok, Poland, for providing us with Polish Valerian root material.

REFERENCES

[1] Houghton PJ. The scientific basis for the reputated activity of valerian. J Pharm Pharmacol 1999;51:505–12.
[2] Ziegler G, Ploch M, Miettinen-Baumann A, Collet W. Efficacy and tolerability of valerian extract LI 156 compared with oxazepam in the treatment of non-organic insomnia—a randomized, double-blind, comparative clinical study. Eur J Med Res 2002;7:480–6.
[3] Wagner J, Wagner ML, Hening WA. Beyond benzodiazepines: alternative pharmacologic agents for the treatment of insomnia. Ann Pharmacother 1998;32:680–91.
[4] Trevana L. Practice corner: sleepless in Sydney—is valerian an effective alternative to benzodiazepines in the treatment of insomnia? ACP J Club 2004;141:A14–6.
[5] Malva JO, Santos S, Macedo T. Neuroprotective properties of *Valeriana officinalis* extracts. Neurotox Res 2004;6:131–40.
[6] Balduini W, Cattabeni F. Displacement of [$^3$H]-N$^6$-cyclohexyladenosine binding to rat cortical membranes by an hydro-alcoholic extract of *Valeriana officinalis*. Med Sci Res 1998;17:639–40.
[7] Müller CE, Schumacher B, Brattström A, Abourashed EA, Kötter U. Interactions of valerian extracts and a fixed valerian-hop extract combination with adenosine receptors. Life Sci 2002;71:1939–49.
[8] Vissiennon Z, Sichardt K, Kötter U, Brattström A, Nieber K. Valerian extract Ze 911 inhibits postsynaptic potentials by activation of adenosine $A_1$ receptors in rat cortical neurons. Planta Med 2006;72:579–83.
[9] Schellenberg R, Sauer S, Abourashed EA, Kötter U, Brattström A. The fixed combination of valerian and hops (Ze 91019) acts via a central adenosine mechanism. Planta Med 2004;70:594–7.
[10] Schumacher B, Scholle S, Hölzl J, Khudeir N, Hess S, Müller CE. Lignans isolated from valerian: identification and characterization of a new olivil derivative with partial agonistic activity at $A_1$ adenosine receptors. J Nat Prod 2002;65:1479–85.
[11] Fredholm BB, Ijzerman AP, Jacobson KA, Klotz K-N, Linden J. International union of pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 2001;53:527–52.
[12] Basheer R, Strecker RE, Thakkar MM, McCarley RW. Adenosine and sleep–wake regulation. Prog Neurobiol 2004;73:379–96.
[13] Marks GA, Birabil CG, Speciale SG. Adenosine $A_1$ receptor mediate inhibition of cAMP formation in vitro in the pontine, REM sleep induction zone. Brain Res 2005;1061:124–7.
[14] Alanko LO, Laitinen JT, Stenberg D, Porkka-Heiskanen T. Adenosine $A_1$ receptor-dependent G-protein activity in the rat brain during prolonged wakefulness. Neuroreport 2004;15:2133–7.
[15] Huang ZL, Qu WM, Eguchi N, Chen JF, Schwarzschild MA, Fredholm BB, et al. Adenosine $A_{2A}$ but not $A_1$ receptors mediate the arousal effect of caffeine. Nat Neurosci 2005;8:858–9.
[16] Yan L, Burbiel JC, Maaß A, Müller CE. Adenosine receptor agonists: from basic medicinal chemistry to clinical development. Expert Opin Emerg Drugs 2003;8:537–76.
[17] Müller CE. $A_1$ adenosine receptor antagonists. Exp Opin Ther Patents 1997; 7:419–40.
[18] Chen JF. The adenosine $A_{2A}$ receptor as an attractive target for Parkinson's disease treatment. Drug News Perspect 2003;16:597–604.
[19] Bigler P. Modern homo- and heteronuclear 1D and 2D NMR experiments: a short overview. In: NMR spectroscopy: processing strategies. Weinheim, Germany: Wiley-VCH; 2000. p. 60–77.
[20] Thies PW, Finner E, David S. Über die Wirkstoffe des Baldrians – XIV. Konstitutive Zuordnung der Acyloxysubstituenten in Valepotriaten via C13-NMR-Spektroskopie (On the bioactive agents of valerian – XIV. Assignment of type and location of the acyloxy

substituents in valepotriates via C13-NMR spectroscopy). Planta Med 1981;41:15–20.
[21] Bos R, Woerdenbag HJ, Pras N. Determination of valepotriates. J Chromatogr A 2002;967:131–46.
[22] Thies PW, Finner E, Rosskopf F. Über die Wirkstoffe des Baldrians – X. Die Konfiguration des Valtratum und anderer Valepotriate. (The configuration of valtratum and other valepotriates). Tetrahedron 1973;29:3213–26.
[23] Handjieva NV, Mollova NN, Popov SS. Chemical ionisation mass spectrometry of valepotriates using isobutane as reactant gas. Planta Med 1987;53:198–201.
[24] Bruns RF, Lu GH, Pugsley TA. Characterization of the adenosine A2 receptor labeled by [$^3$H]NECA in rat striatal membranes. Mol Pharmacol 1986;29:331–46.
[25] Lohse MJ, Lenschow V, Schwabe U. Interaction of barbiturates with adenosine receptors in rat brain. Nauyn Schmiedeberg's Arch Pharmacol 1984;326:69–74.
[26] Klotz K-N, Lohse MJ, Schwabe U, Cristalli G, Vittori S, Grifantini M. 2-Chloro-$N^6$-[$^3$H]cyclopentyladenosine ([$^3$H]CCPA)—a high affinity agonist radioligand for $A_1$ adenosine receptors. Nauyn Schmiedeberg's Arch Pharmacol 1989;340:679–83.
[27] Müller CE, Maurinsh J, Sauer R. Binding of [$^3$H]MSX-2(3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine) to rat striatal membranes. Eur J Pharm Sci 2000;10:259–65.
[28] Lorenzen A, Guerra L, Vogt H, Schwabe U. Interaction of full and partial agonists of the $A_1$ adenosine receptor with receptor/G-protein complexes in rat brain membranes. Mol Pharmacol 1996;49:915–26.
[29] Klotz K-N, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB, et al. Comparative pharmacology of human adenosine receptor subtypes—characterization of stably transfected receptors in CHO cells. Nauyn Schmiedeberg's Arch Pharmacol 1998;357:1–9.
[30] Brand A, Vissiennon Z, Eschke D, Nieber K. Adenosine $A_1$ and $A_3$ receptors mediate inhibition of synaptic transmission in rat cortical neurons. Neuropharmacology 2001;40:85–95.
[31] Hentschel S, Lewerenz A, Nieber K. Activation of $A_3$ receptors by endogenous adenosine inhibits synaptic transmission during hypoxia in rat cortical neurons. Restor Neurol Neurosci 2003;21:55–63.
[32] Weyler S, Fülle F, Diekmann M, Schumacher B, Hinz S, Klotz K-N, et al. Improving potency, selectivity, and water solubility of adenosine $A_1$ receptor antagonists: xanthines modified at position 3 and related pyrimido[1,2,3-*cd*]purinediones. Chem Med Chem 2006;1:891–902.
[33] Thies PW. Zur Chemie der Valepotriate. 4. Mitteilung über die Wirkstoffe des Baldrians (on the chemistry of valepotriates. 4. Report on the bioactive constituents of valerian). Deutsche Apotheker Zeitung 1967;107:1411–2.
[34] Daly JW, Hide I, Müller CE, Shamim M. Caffeine analogs: structure–activity relationships at adenosine receptors. Pharmacology 1991;42:309–21.