European Journal of Pharmacology 735 (2014) 123–131

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Behavioural pharmacology

Esters of valerenic acid as potential prodrugs

Juliane Hintersteiner [a], Maximilian Haider [b], Denise Luger [a], Christoph Schwarzer [c], Gottfried Reznicek [d], Walter Jäger [e], Sophia Khom [a], Marko D. Mihovilovic [b], Steffen Hering [a,*]

[a] Department of Pharmacology and Toxicology, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria
[b] Institute of Applied Synthetic Chemistry, Vienna University of Technology, Getreidemarkt 9, A-1060 Vienna, Austria
[c] Department of Pharmacology, Innsbruck Medical University, Peter-Mayr-Straße 1, 1a A-6020 Innsbruck, Austria
[d] Department of Pharmacognosy, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria
[e] Department of Clinical Pharmacy and Diagnostics, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria

ARTICLE INFO

Article history:
Received 9 December 2013
Received in revised form
7 March 2014
Accepted 19 March 2014
Available online 27 March 2014

Keywords:
$GABA_A$ receptors
Valerenic acid derivatives
Behavioral analysis
2-Microelectrode-voltage-clamp-technique
LC–MS/MS

ABSTRACT

Valerenic acid (VA) is a $\beta_{2/3}$ subunit-specific modulator of γ-aminobutyric acid (GABA) type A ($GABA_A$) receptors inducing anxiolysis. Here we analyze if VA-esters can serve as prodrugs and if different ester structures have different in vitro/in vivo effects. Modulation of $GABA_A$ receptors expressed in *Xenopus* oocytes was studied with 2-microelectrode-voltage-clamp. Anxiolytic effects of the VA-esters were studied on male C57BL/6N mice by means of the elevated plus maze-test; anticonvulsant properties were deduced from changes in seizure threshold upon pentylenetetrazole infusion. VA was detected in plasma confirming hydrolysis of the esters and release of VA in vivo. Esterification significantly reduced the positive allosteric modulation of $GABA_A$ ($\alpha_1\beta_3\gamma_{2S}$) receptors in vitro. in vivo, the studied VA-ester derivatives induced similar or even stronger anxiolytic and anticonvulsant action than VA. While methylation and propylation of VA resulted in faster onset of anxiolysis, the action of VA-ethylester was longer lasting, but occurred with a significant delay. The later finding is in line with the longer lasting anticonvulsant effects of this compound. The estimated VA plasma concentrations provided first insight into the release kinetics from different VA-esters. This might be an important step for its future clinical application as a potential non-sedative anxiolytic and anticonvulsant.

1. Introduction

γ-Aminobutyric acid (GABA) type A receptors ($GABA_A$) are the major inhibitory neurotransmitter receptors in the mammalian brain. $GABA_A$ receptors belong to the superfamily of Cys-loop-type ligand-gated ion channels (Olsen and Sieghart, 2008). Nineteen $GABA_A$ receptor subunits have been identified in the human genome, comprising α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, π and ρ_{1-3} (Simon et al., 2004). Five receptor subunits form a chloride-selective ion channel. Receptor activation opens the channel and induces transmembrane chloride currents (I_{GABA}) modulating neuronal excitability and transmitter release (Sieghart, 2006; Sigel and Steinmann, 2012). There is consensus that the major adult receptor isoform consists of $2\alpha_1$, $2\beta_2$ and one γ_2 subunit (Olsen and Sieghart, 2008).

$GABA_A$ receptors play a major role in the treatment of central nervous system (CNS) diseases such as generalized anxiety and panic disorders, epilepsy, and sleep disturbances (Möhler, 2006). They are the molecular target of the classical benzodiazepines (e.g. diazepam) and subtype-selective benzodiazepine site ligands such as zolpidem or zopiclone, barbiturates, anaesthetics, and anticonvulsants (Sigel and Steinmann, 2012). Beside these drugs, $GABA_A$ receptors are modulated by multiple natural products (Johnston et al., 2006).

We and others have shown that valerenic acid (VA), a constituent of *Valeriana officinalis*, enhances I_{GABA} through $GABA_A$ receptors. VA binds with nanomolar affinity (Benke et al., 2009) and modulates $GABA_A$ receptors in an allosteric manner. VA selectively interacts with receptors comprising $\beta_{2/3}$-subunits (Benke et al., 2009; Khom et al., 2007). A point mutation in the β_2-subunit (N265S) of recombinant receptors prevents I_{GABA}

* Corresponding author. Tel.: +43 1 4277 55301/55310; fax: +43 1 4277 9553.
E-mail addresses: juliane.hintersteiner@univie.ac.at (J. Hintersteiner), maximilian.haider@univie.ac.at (M. Haider), denise.luger@univie.ac.at (D. Luger), schwarzer.christoph@i-med.ac.at (C. Schwarzer), gottfried.reznicek@univie.ac.at (G. Reznicek), walter.jaeger@univie.ac.at (W. Jäger), sophia.khom@univie.ac.at (S. Khom), marko.mihovilovic@tuwien.ac.at (M.D. Mihovilovic), steffen.hering@univie.ac.at (S. Hering).

http://dx.doi.org/10.1016/j.ejphar.2014.03.019
0014-2999/

enhancement while the "reversed mutation" in β_1 (S266N) enhances current stimulation to extents observed on $\beta_{2/3}$-subunit containing receptors (Khom et al., 2007). Valerenic acid induces anxiolysis in the elevated plus maze and the light/dark choice test in mice (Benke et al., 2009; Khom et al., 2010). Anxiolysis was absent in β_3(N265M) point-mutated mice supporting the hypothesis that the anxiolytic effects of VA are caused by interaction with β_3-containing $GABA_A$ receptors (Benke et al., 2009).

A recently published pharmacokinetic study on rats revealed that approximately 34% of VA are absorbed after oral administration with a half-life between 2.7 and 5 h (Sampath et al., 2012). This good bioavailability is in line with the reported anxiolysis of VA after oral administration in mice (Benke et al., 2009).

Together these findings make VA or one of its derivatives (Khom et al., 2010; Kopp et al., 2010) interesting drug candidates. Little is known, however, how this molecule penetrates the blood-brain barrier (Neuhaus et al., 2008). Ester prodrugs can enhance the lipophilicity (by masking charged groups such as carboxylic acids) and thereby affect the time course of drug action (Beaumont et al., 2003). Therefore four VA-esters (VA-methylester (VA-ME), VA-ethylester (VA-EE), VA-propylester (VA-PE) and VA-pivaloyloxymethylester (VA-POM)) have been synthesized in order to address the following questions about the biological activity of these potential prodrugs: (i) Does esterification affect modulation of I_{GABA} through $GABA_A$ receptors? (ii) Do VA-esters represent prodrugs (i.e. are esters hydrolyzed in vivo and is VA detectable in the plasma? (iii) Are VA-esters active in vivo and – if so – does esterification affect the anxiolytic and anticonvulsant properties of VA?

2. Materials and methods

All experiments on animals were carried out in accordance to the Austrian Animal Experimental Law, which is in line with the EU directive 2010/63/EU.

2.1. Chemicals

Valerenic acid (VA) was purchased from HWI Pharma Solutions (Rülzheim, Germany) and converted into the aforementioned derivatives as described below (for structural formulae see Fig. 1). Chemicals used in this study were obtained from Sigma-Aldrich (Vienna, Austria) except where otherwise stated. Dichloromethane (DCM), dimethylsulfoxide (DMSO), formic acid, methanol and *t*-butylmethylether were of p.a. quality and purchased from ROTH (Karlsruhe, Germany). For HPLC analysis double distilled water and acetonitrile, HPLC quality (VWR Int., Vienna, Austria) were used.

LogP values for the aimed compounds were calculated using ACD/ChemSketch freeware.

All reactions were carried out in oven dried 4 ml-reaction vials under an argon atmosphere. DCM was predistilled and then desiccated on Al_2O_3 columns (PURESOLV, Innovative Technology; Amesbury, USA). Reaction mixtures were magnetically stirred and monitored by thin layer chromatography using Merck Silica $60F_{254}$ plates (Merck, Vienna, Austria). Flash chromatography was performed on a Sepacore Flash System (2 × Büchi Pump Module C-605, Büchi Pump Manager C-615, Büchi UV Photometer C-635, Büchi Fraction Collector C-660; Büchi Labortechnik, Flawil, Switzerland) using Merck silica gel (0.040–0.063 mm, 230–400 mesh). Yields refer to chromatographically and spectroscopically pure compounds. ^{1}H-NMR (200 MHz) and ^{13}C-NMR (50 MHz) were recorded on Bruker AC 200 (200 MHz; Bruker, Karlsruhe, Germany). The chemical shifts δ are reported relative to the residual solvent peaks. All ^{1}H and ^{13}C shifts are given in ppm (s=singulet, d=doublet, t=triplet, q=quadruplet, m=multiplet). Specific rotation was measured on an Anton Paar MCP500 polarimeter (Anton Paar GmbH; Graz, Austria) at 20 °C in DCM.

LC–MS/MS analyses were carried out on an Ultimate 3000 RSLC-series system (Thermo Fisher Scientific Austria, Vienna, Austria) coupled to a triple quadrupol mass spectrometer API 4000 (AB Sciex Instruments, Framingham, USA).

2.2. Synthesis of valerenic acid esters

2.2.1. Valerenic acid methylester (VA-ME)

Valerenic acid (30.0 mg, 1 Eq., 0.13 mmol) and 4-dimethylaminopyridine (DMAP, 1.6 mg, 0.1 Eq, 0.01 mmol) were dissolved in 1.3 ml dry DCM under an Argon atmosphere and cooled to 0 °C, then 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI; 36.8 mg, 1.5 Eq, 0.19 mmol) was added in one portion. After stirring the mixture for five min methanol (23.7 µl, 4.5 Eq, 0.59 mmol) was added dropwise and the mixture was left warming to room temperature overnight. The solution was taken up in 50 ml ethylacetate (EtOAc) and was subsequently washed with saturated NH_4Cl solution (three times), saturated $NaHCO_3$ solution (three times) and once with brine; it was then dried and concentrated under reduced pressure. Purification of the crude material via column chromatography (LP:EtOAc=30:1) provided 30.2 mg (94%) of Valerenic acid methylester as a colorless oil.

- **^{1}H-NMR (200 MHz, $CDCl_3$):** δ=0.77 (d, J_1=7.0, 3*H*), 1.37–1.98 (m, 14*H*), 2.19 (t, J_1=7.5 Hz, 1*H*), 2.90–2.99 (m, 1*H*), 3.49–3.56 (m, 1*H*), 3.72 (s, 3*H*), 7.01 (dq, J_1=1.4 Hz, J_2=9.8 Hz, 1*H*)
- **^{13}C-NMR (50 MHz, $CDCl_3$)** δ=12.0 (s), 12.4 (s), 13.5 (s), 24.5 (d), 25.4 (d), 28.7(d), 33.0 (t), 34.3 (t), 37.4 (d), 47.4 (t), 51.7 (s), 125.7 (q), 130.9 (q), 133.4 (t), 169.0 (q)

Analytical data is consistent with the reported data for Valerenic acid methylester (Kopp et al., 2010).

2.2.2. Valerenic acid ethylester (VA-EE)

Using the analogous procedure as for the preparation of VA-ME, treatment of valerenic acid (20.0 mg, 1 Eq, 0.09 mmol) with 4-dimethylaminopyridine (1.0 mg, 0.1 Eq, 0.009 mmol), EDCI (24.4 mg, 1.5 Eq, 0.13 mmol) and ethanol (22.3 µl, 4.5 Eq, 0.38 mmol) yielded 20.2 mg (95%) of VA-EE as colorless oil.

- **^{1}H-NMR (200 MHz, $CDCl_3$, ppm):** δ=0.78 (d, J=7.0, 3*H*), 1.29 (t, J=7.1 Hz, 3*H*), 1.37–2.02 (m, 14*H*), 2.19 (t, J=7.6 Hz, 2*H*), 2.92–2.98 (m, 1*H*), 3.46–3.56 (m, 1*H*), 4.17 (q, J=7.1 Hz, 2*H*), 7.01 (dq, J_1=9.8 Hz, J_2=1.4 Hz, 1*H*)
- **^{13}C-NMR (50 MHz, $CDCl_3$, ppm):** δ=12.0 (s), 12.4 (s), 13.5 (s), 14.3 (s), 24.5 (d), 25.5 (d), 28.7(d), 33.1 (t), 34.3 (t), 37.4 (d), 47.4 (t), 60.4 (d), 125.7 (q), 130.9 (q), 133.4 (t), 169.0 (q)

Analytical data is consistent with the reported data for Valerenic acid ethylester (Kopp et al., 2010).

2.2.3. Valerenic acid propylester (VA-PE)

Using the analogous procedure as for the preparation of VA-ME, treatment of valerenic acid (30.0 mg, 1 Eq, 0.13 mmol) with 4-DMAP (1.6 mg, 0.1 Eq, 0.01 mmol), EDCI (36.8 mg, 1.5 Eq, 0.19 mmol) and propanol (28.5 µl, 4.5 Eq, 0.38 mmol) yielded 35.1 mg (99%) of VA-PE as colorless oil.

- **^{1}H-NMR (200 MHz, $CDCl_3$, ppm):** δ=0.77 (d, J=7.0, 3*H*), 0.95 (t, J=7.4 Hz, 3*H*), 1.37–2.01 (m, 16*H*), 2.19 (t, J=7.7 Hz, 2*H*), 2.91–2.97 (m, 1*H*), 3.48–3.55 (m, 1*H*), 4.07 (t, J=6.7 Hz, 2*H*), 7.02 (dq, J_1=9.8 Hz, J_2=1.3 Hz, 1*H*)