Pharmacological Research 80 (2014) 52–65

Contents lists available at ScienceDirect

Pharmacological Research

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

Guineensine is a novel inhibitor of endocannabinoid uptake showing cannabimimetic behavioral effects in BALB/c mice

Simon Nicolussi[a], Juan Manuel Viveros-Paredes[b], María Salomé Gachet[a], Mark Rau[a], Mario Eduardo Flores-Soto[b], Martina Blunder[c], Jürg Gertsch[a,*]

[a] Institute of Biochemistry and Molecular Medicine, NCCR TransCure, University of Bern, CH-3012 Bern, Switzerland
[b] Departamento de Farmacobiología CUCEI, Universidad de Guadalajara, 44430 Guadalajara, Jalisco, Mexico
[c] Department of Neuroscience, Uppsala University, BMC, 751 24 Uppsala, Sweden

ARTICLE INFO

Article history:
Received 29 October 2013
Received in revised form 4 December 2013
Accepted 31 December 2013

Keywords:
Guineensine
Anandamide uptake
Endocannabinoid transport inhibition
Cannabimimetic
Endocannabinoid system

Chemical compounds studied in this article:
Guineensine (PubChem CID: 6442405)
Anandamide (PubChem CID: 5281969)
2-Arachidonoylglycerol (PubChem CID: 5282280)
OMDM-2 (PubChem CID: 57347656)
UCM707 (PubChem CID: 53394011)
URB597 (PubChem CID: 1383884)
JZL184 (PubChem CID: 25021165)
Haloperidol (PubChem CID: 3559)
(+)-R-WIN 55,212-2 (PubChem CID: 5311501)
Rimonabant (PubChem CID: 104850)

ABSTRACT

High-content screening led to the identification of the *N*-isobutylamide guineensine from *Piper nigrum* as novel nanomolar inhibitor (EC_{50} = 290 nM) of cellular uptake of the endocannabinoid anandamide (AEA). Noteworthy, guineensine did not inhibit endocannabinoid degrading enzymes fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL) nor interact with cannabinoid receptors or fatty acid binding protein 5 (FABP5), a major cytoplasmic AEA carrier. Activity-based protein profiling showed no inhibition of serine hydrolases. Guineensine also inhibited the cellular uptake of 2-arachidonoylglycerol (2-AG). Preliminary structure–activity relationships between natural guineensine analogs indicate the importance of the alkyl chain length interconnecting the pharmacophoric isobutylamide and benzodioxol moieties for AEA cellular uptake inhibition. Guineensine dose-dependently induced cannabimimetic effects in BALB/c mice shown by strong catalepsy, hypothermia, reduced locomotion and analgesia. The catalepsy and analgesia were blocked by the CB1 receptor antagonist rimonabant (SR141716A). Guineensine is a novel plant natural product which specifically inhibits endocannabinoid uptake in different cell lines independent of FAAH. Its scaffold may be useful to identify yet unknown targets involved in endocannabinoid transport.

Abbreviations: AA, arachidonic acid; ABHD, alpha/beta hydrolase; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; BSA, bovine serum albumin; $CHCl_3$, chloroform; CB1, cannabinoid receptor type-1; CB2, cannabinoid receptor type-2; CNS, central nervous system; CPM, counts per minute; COX, cyclooxygenase; DMSO, dimethyl sulphoxide; DQF-COSY, double quantum filtered correlation spectroscopy; EC_{50} = relative IC_{50}, the molar concentration of an inhibitor that corresponds to the half maximal (50%) effect midway between the upper and lower plateaus; EC, endocannabinoid; ECS, endocannabinoid system; EtNH2, ethanolamine; EtOAc, ethylacetate; FAAH, fatty acid amide hydrolase; FABP5, fatty acid binding protein 5; FP, fluorescence polarization; HMC-1, human leukemia mast cells; HMBC, Heteronuclear Multiple Bond Correlation; HPLC, high pressure liquid chromatography; HSQC, Heteronuclear Single Quantum Coherence; IC_{50} = absolute IC50, the molar concentration of an inhibitor that corresponds to the half maximal (50%) of control response (the mean of the 0% and 100% assay controls); LOX, lipoxygenase; MAGL, monoacylglycerol lipase; NMR, nuclear magnetic resonance spectroscopy; SPE, solid phase extraction; SAR, structure–activity relationship; U937, human monocytic lymphoma cell line; TLC, thin layer chromatography.

* Corresponding author at: Institute of Biochemistry and Molecular Medicine, NCCR TransCure, University of Bern, Bühlstrasse 28, CH-3012 Bern, Switzerland. Tel.: +41 031 631 41 24; fax: +41 031 631 37 37.
E-mail address: gertsch@ibmm.unibe.ch (J. Gertsch).

1043-6618/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.phrs.2013.12.010

1. Introduction

The endocannabinoid system (ECS) is a lipid signaling system comprising endocannabinoids (ECs), which are lipids derived from arachidonic acid, the G-protein-coupled cannabinoid receptors CB1 and CB2, as well as several other actual and potential physiological targets involved in the synthesis, transport and degradation of ECs [1–3]. The major ECs are 2-arachidonoylglycerol (2-AG) and *N*-arachidonoyl ethanolamide (AEA) which modulate synaptic transmission by retrograde signaling *via* CB1 receptors and exert potent immunomodulatory effects *via* both CB1 and CB2 receptors [4–6]. The ECS has been implicated in physiological and pathophysiological conditions including inflammation, pain, psychiatric disorders and metabolic reprogramming [1,7–9]. Therapeutic strategies within the ECS include the use of cannabinoid receptor agonists and antagonists, blockage of hydrolytic enzymes degrading ECs such as fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), as well as inhibition of EC membrane trafficking [1,10–12]. The latter approach is less well established and there is a debate about whether EC cell membrane transport is regulated by a putative membrane transporter or cytoplasmic carrier proteins [13–17]. Here we searched for novel small organic molecules able to inhibit cellular anandamide (AEA) uptake but do not affect hydrolytic enzymes, such as the serine hydrolase FAAH. FAAH generates an inward concentration gradient for AEA, which is the major driving force for its cellular uptake [18]. Therefore, FAAH is a major target to be taken into account during AEA uptake measurements [19]. Although so far no membrane protein for EC transport has been identified, several lines of evidence suggest a facilitated membrane transport involving both membrane and cytoplasmic targets [13,15–17,20–22]. Using the commercially available AEA uptake inhibitors UCM707, OMDM-2 and LY2183240 [22–24] we recently provided evidence for bidirectional transport of both AEA and 2-AG across cell membranes, as well as a common mechanism of cellular membrane transport for all arachidonate-based ECs [13]. All inhibitors were also able to reduce AEA efflux, an effect that cannot be explained by inhibition of cytoplasmic carrier proteins. Since all of the available inhibitors are relatively weak in potency (IC_{50} in the low micromolar range) and/or show low selectivity toward AEA transport inhibition over FAAH inhibition or other cytoplasmic targets, the elucidation of the mechanisms of AEA and 2-AG uptake is hampered by a lack of adequate tools [14,15,25,26]. There is a need for novel inhibitors of EC transport with superior specificity as novel probes which may help to better understand the mechanisms of EC cell membrane transport.

Pepper species (*Piper* spp.) exhibit a rich phytochemistry with numerous bioactive scaffolds reported, including 3,4-methylenedioxyphenylpropan-2-one metabolites [27–29]. In Asian traditional medicine pepper species have been used for millennia and are known to exert analgesic and anti-inflammatory effects [29–31]. Moreover, *Piper nigrum* L. and *Piper longum* L. are used traditionally to treat psychiatric disorders like affective disorders [32,33]. In an iterative screening and bioactivity-guided isolation we have identified *P. nigrum* L. and *P. longum* L. as positive hit extracts in AEA uptake inhibition. Subsequent bioactivity-guided fractionation and isolation led to the discovery of guineensine as the first plant natural product able to potently inhibit cellular EC uptake showing central cannabimimetic effects in BALB/c mice.

2. Materials and methods

2.1. Materials

Compounds and chemicals were of purest possible grade. Anandamide (AEA), 2-arachidonoylglycerol (2-AG), *(R)-N*-(1-(4-hydroxyphenyl)-2-hydroxyethyl)oleamide (OMDM-2), *N*-(3-furanylmethyl)-(*5Z,8Z,11Z,14Z*)-eicosatetraenamide (UCM 707), (3-aminocarbonyl)[1,1-biphenyl]-3-yl)-cyclohexylcarbamate (URB597), methyl-arachidonyl-fluorophosphonate (MAFP) and the COX fluorescent inhibitor screening assay kit were purchased from Cayman Chemicals Europe. Analytical grade reagents (*n*-hexane, EtOAc, chloroform, acetone, dichloromethane, cyclohexane and methanol) were supplied by Grogg Chemie AG, Switzerland. HPLC-grade acetonitrile was obtained from (Sigma–Aldrich Chemie GmbH, Germany). 4-[*Bis*(1,3-benzodioxol-5-yl)hydroxy-methyl]-1-piperidinecarboxylic acid 4-nitro-phenylester (JZL184) and (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-*yl*]-1-naphthalenyl methanone mesylate (*R*-(+)-WIN55,212-2) were purchased from Tocris Bioscience. 12-*N*-methyl-(7-nitrobenz-2-oxa-1,3-diazo)aminostearic acid (NBD-Stearate) was obtained from Avanti Polar Lipids. CP55,940 [side chain-2,3,4-^{3}H(N)] (144 Ci/mmol) was ordered from PerkinElmer. [*ethanolamine*-1-^{3}H]-AEA (60 Ci/mmol), [*arachidonoyl*-5,6,8,9,11,12,14,15-^{3}H]-AEA (200 Ci/mmol), [*arachidonoyl*-5,6,8,9,11,12,14,15-^{3}H]-2-AG (200 Ci/mmol), [*glycerol*-1,2,3-^{3}H]-2-OG (60 Ci/mmol) were purchased from American Radiolabeled Chemicals. Albumin from bovine serum essentially fatty acid free (BSA) (A7030), fetal bovine serum (F7524), RPMI-1640, Iscove's Modified Dulbecco's Medium, monothioglycerol, FABP5 (human recombinant), rofecoxib and LPS (E.coli 055:B5) were purchased from Sigma-Aldrich, Germany. AquaSil™ siliconizing fluid and the ActivX® TAMRA-FP Serine Hydrolase Probe (88318) were purchased from Thermo Scientific. Rimonabant (SR141716A) was purchased from pharmaserv AG, Switzerland. Inhepar® was obtained from Pisa Pharmaceutica, Mexico. Haloperidol (Haldol®) was purchased as injectable solution of 5 mg/mL from Janssen-Cilag, Switzerland.

2.2. Cell culture

U937 human monocytic leukemia cells were purchased from American Type Culture Collection (Manassas, VA) and were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 12.5 μg/mL amphotericin B, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Sigma–Aldrich). HMC-1 human mast cells were a kind gift of Prof. Dr. S. Ständer, University of Münster, Germany and were cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% FBS (F7524), 100 U/mL penicillin, 100 μg/mL streptomycin and monothioglycerol (10 μM). Cells were cultured in an incubator at 37 °C with a 5% CO_2 humidified atmosphere. CHO-K1 cells (ATCC) stably transfected with hCB_1 or hCB_2 were grown in RPMI 1640 medium, 10% FBS supplemented with 12.5 μg/mL amphotericin B, 100 U/mL penicillin and 100 μg/mL streptomycin and 160 μL/10 mL Geneticin G418. Cells were grown up to 90% confluence before being harvested for membrane preparation.

2.3. Preparation of extracts for AEA uptake inhibition screening

Screening extracts were produced by accelerated solvent extraction (Dionex ASE 300) using EtOAc covering a medium-polarity range. 50 g of dried plant material was extracted automatically with 200 mL EtOAc in 3 cycles, 70 °C, 100 bar. Dry extracts resulted in yields of 1.2–9.3%. Extracts were dissolved in DMSO to stock solutions at 250 μg/mL in Eppendorf 96 deep-well blocks ready for screening.

2.4. Extraction and isolation

Dry fruits of *P. nigrum* L. were obtained from Delica AG, Switzerland. The powdered fruits (1.05 kg) were extracted subsequently by percolation with *n*-hexane, $CHCl_3$ and CH_3OH to