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Correlating FAAH and anandamide cellular uptake inhibition using *N*-alkylcarbamate inhibitors: From ultrapotent to hyperpotent

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ABSTRACT

Besides the suggested role of a putative endocannabinoid membrane transporter mediating the cellular uptake of the endocannabinoid anandamide (AEA), this process is intrinsically coupled to AEA degradation by the fatty acid amide hydrolase (FAAH). Differential blockage of each mechanism is possible using specific small-molecule inhibitors. Starting from the natural product-derived 2*E*,4*E*-dodecadiene scaffold previously shown to interact with the endocannabinoid system (ECS), a series of diverse *N*-alkylcarbamates were prepared with the aim of generating novel ECS modulators. While being inactive at cannabinoid receptors and monoacylglycerol lipase, these *N*-alkylcarbamates showed potent to ultrapotent picomolar FAAH inhibition in U937 cells. Overall, a highly significant correlation (Spearman's rho = 0.91) was found between the inhibition of FAAH and AEA cellular uptake among 54 compounds. Accordingly, in HMC-1 cells lacking FAAH expression the effect on AEA cellular uptake was dramatically reduced. Unexpectedly, 3-(4,5-dihydrothiazol-2-yl)phenyl carbamates and the 3-(1,2,3-thiadiazol-4-yl)phenyl carbamates WOBE490, WOBE491 and WOBE492 showed a potentiation of cellular AEA uptake inhibition in U937 cells, resulting in unprecedented femtomolar (hyperpotent) IC_{50} values. Potential methodological issues and the role of cellular accumulation of selected probes were investigated. It is shown that albumin impacts the potency of specific *N*-alkylcarbamates and, more importantly, that accumulation of FAAH inhibitors can significantly increase their effect on cellular AEA uptake. Taken together, this series of *N*-alkylcarbamates shows a FAAH-dependent inhibition of cellular AEA uptake, which can be strongly potentiated using specific head group modifications. These findings provide a rational basis for the development of hyperpotent AEA uptake inhibitors mediated by ultrapotent FAAH inhibition.

1. Introduction

The arachidonic acid-derived lipid anandamide (AEA, arachidonoyl ethanolamide) is a primary endocannabinoid, which is synthesized on demand and released by cells upon stimulation [1]. Among the different cells known to produce endocannabinoids, neurons and immune cells are best studied [1–5]. Extracellular AEA can activate both CB_1 and CB_2 cannabinoid G protein-coupled receptors, but has also intracellular targets, such as TRPV1, potassium and calcium channels, as well as PPARs [6]. The cellular uptake of AEA is driven by a gradient that is generated by its major metabolizing enzyme fatty acid amide hydrolase (FAAH), which cleaves AEA to arachidonic acid (AA) and ethanolamine ($EtNH_2$) [5,7,8]. FAAH is a serine hydrolase expressed primarily in the endoplasmic reticulum [9–13]. In conditions where COX-2 is expressed, AEA is also oxygenated by this enzyme [14,15]. Moreover, under certain circumstances cytochrome P450 enzymes may metabolize AEA [16,17]. The relationship between FAAH activity and AEA cellular uptake has been described already several years

Abbreviations: AA, arachidonic acid; AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; BSA, bovine serum albumin; $CHCl_3$, chloroform; CB_1, cannabinoid receptor type-1; CB_2, cannabinoid receptor type-2; COX, cyclooxygenase; DMSO, dimethyl sulfoxide; ECs, endocannabinoids; ECS, endocannabinoid system; $EtNH_2$, ethanolamine; FAAH, fatty acid amide hydrolase; FABP5, fatty acid binding protein 5; HMC-1, human mast cell leukemia cells; IC_{50}, half maximal (50%) inhibitory concentration; LSC, liquid scintillation counting; MAGL, monoacylgylcerol lipase; PPAR, peroxisome proliferator-activated receptor; SAR, structure–activity relationship; U937, human monocytic lymphoma cell line.

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ago, but it remains unclear whether yet unknown proteins in the membrane and cytoplasm are involved in the physiological process of cell membrane AEA transport [8,9,18–24]. For example, different cytoplasmic AEA binding proteins have been reported to regulate cytoplasmic trafficking and compartmentalization of AEA, which may affect its cellular uptake [25–31]. In addition to the AEA metabolizing enzymes and cytoplasmic carrier proteins, several lines of evidence suggest the existence of a facilitated membrane transport mechanism that mediates the cellular uptake of AEA, 2-arachidonoylglycerol (2-AG) and possibly other endocannabinoids in certain cell types [21,22,24,32–41]. To date, different AEA uptake inhibitors have been published and most of them are commercially available, such as AM404, VDM11, AM1172 and LY2183240 [37,38,40,42]. However, these inhibitors are marginally specific as they also inhibit FAAH activity with similar potencies at which they interfere with the endocannabinoid trafficking across plasma membranes [11,43–45]. At high concentrations, these AEA cellular uptake inhibitors inhibit AEA uptake through FAAH inhibition [7,22,43] and may interact also with intracellular carrier proteins such as FABP5 [46]. Nevertheless, using some of the above mentioned inhibitors, independent studies have provided evidence in favor of a putative endocannabinoid membrane transporter (EMT) in electrophysiological studies in neurons, where extracellular AEA, but also 2-AG levels were elevated [41,47]. Additional evidence of a facilitated membrane transport was provided with VDM11 and the more selective AEA uptake inhibitors OMDM-2 and UCM707, showing inhibition of the cellular efflux of AEA and 2-AG [21,22]. Recently, we have reported the biochemical and pharmacological characterization of the new AEA uptake inhibitor guineensine (IC_{50} = 290 nM) [34]. This natural *N*-isobutylamide reversibly inhibits AEA cellular uptake in both U937 cells that strongly express FAAH and HMC-1 cells lacking FAAH expression, but without targeting FABP5 and the other known proteins of the ECS [34].

Although in principle all FAAH inhibitors are able to inhibit the cellular uptake of AEA through depletion of the gradient generated by metabolism, the underlying mechanistic relationship is still insufficiently studied. For instance, it cannot *a priori* be excluded that at least some serine hydrolase-active carbamates could also target other proteins involved in AEA transport, a hypothesis addressed in this study. Furthermore, a FAAH inhibitor needs to pass the cell membrane to block AEA uptake and some inhibitors may not enter the cell just by simple diffusion. While numerous potent reversible and irreversible FAAH inhibitors have been reported in the literature (i.e. URB597 $IC_{50} = 4.6 \pm 1.6$ nM [48], IC_{50} = 3.8 nM (95% CI 2.9–5.0 nM) [49] or LY2183240 IC_{50} = 12.4 nM [43], IC_{50} = 2.1 nM [50], as well as *N*-alkylcarbamates [51–53], a systematic assessment of the expected positive association between (irreversible) FAAH inhibition and AEA cellular uptake is lacking. To that aim, a series of 54 new *N*-alkylcarbamate probes was synthesized based on the *Echinacea* plant-derived 2*E*,4*E*-dodecadieneisobutylamide scaffold, a bioactive natural product known to interact with CB_2 receptors [54]. Related natural *N*-alkylamides appear to weakly inhibit FAAH and AEA cellular uptake [35,55]. By modification of the natural amide group we have generated synthetic *N*-alkylcarbamates which are simple probes that constitute different alkyl chains required for the binding to the hydrophobic channel of FAAH [13,56] combined with distinct head groups involved in the heterolytic bond cleavage. Both, FAAH inhibition and the blockage of AEA cellular uptake by small molecule inhibitors have been shown to exert therapeutic effects in animal models of pain, inflammation, neuropsychiatric diseases, and pruritus [57–62]. The mechanistic and ultimately pharmacological relationship of such inhibitors are therefore of interest also in the context of drug discovery and development.

In this study, *N*-alkylcarbamate FAAH inhibitors were investigated by using well-characterized AEA transport/uptake assays in U937 and HMC-1 cells as previously reported [22,34,63–65]. This analysis led to the discovery that certain probes do not enter the cell just by simple diffusion but transport, which may enable a hitherto unknown dimension of potency enhancement for cellular FAAH and AEA uptake inhibition.

2. Materials and methods

2.1. Chemicals and materials

Compounds and chemicals were of purest possible grade. Anandamide (AEA), 2-arachidonoylglycerol (2-AG), BMS309403, diclofenac, indomethacin, LY2183240, URB597, R-(+)-WIN55,212-2, human recombinant FAAH (*h*FAAH) and the COX fluorescent inhibitor screening assay kit were purchased from Cayman Europe, Estonia. Guineensine was isolated from black pepper (*Piperis nigri fructus*) according to Nicolussi et al. [34]. Radiolabeled CP55,940 [side chain-2,3,4-^{3}H(N)] (144 Ci/mmol), GF/B and GF/C filter plates and MicroScint 20 were ordered from Perkin-Elmer, Switzerland. [ethanolamine-1-^{3}H]-AEA (60 Ci/mmol) and [glycerol-1,2,3-^{3}H]-2-OG (60 Ci/mmol) were purchased from American Radiolabeled Chemicals Inc., USA. Albumin from bovine serum essentially fatty acid free (BSA) (A7030), dipyridamole, fetal bovine serum (F7524), Iscove's Modified Dulbecco's Medium, MK-571, monothioglycerol, RPMI-1640 and verapamil were purchased from Sigma–Aldrich, Switzerland. AquaSil™ siliconizing fluid was purchased from Thermo Fisher Scientific, USA. Endocannabinoids were regularly checked by LC–MS/MS for purity.

Commercially available starting materials for carbamate synthesis were used without further purification. All chemicals were provided from MercaChem, the Netherlands. The following starting material were prepared according to the literature [66,67]: (*2E,4E*)-dodeca-2,4-dien-1-amine, tert-butyl (*2E,4E*)-dodeca-2,4-dienylcarbamate, (*2E,4E*)-7-phenylhepta-2,4-dien-1-amine, (*2E,4E*)-8-phenylocta-2,4-dien-1-amine, (*2E,4E*)-7-(pyridin-3-yl)hepta-2,4-dien-1-amine, (*2E,4E*)-7-butoxyhepta-2,4-dien-1-amine hydrochloride, (*2E,4E*)-6-(pentyloxy)hexa-2,4-dien-1-amine, (*2E,4E*)-N-methyldodeca-2,4-dien-1-amine hydrochloride, (*2E,4E*)-N-methyl-7-phenylhepta-2,4-dien-1-amine hydrochloride, (*2E,4E*)-7-butoxy-N-methylhepta-2,4-dien-1-amine hydrochloride, (*2E,4E*)-N-methyl-6-(pentyloxy)hexa-2,4-dien-1-amine hydrochloride, 5-(hydroxymethyl)isoxazole-3-carboxamide, 5-(hydroxymethyl)-N-methylisoxazole-3-carboxamide, 5-(hydroxymethyl)-N,N-dimethylisoxazole-3-carboxamide.

2.2. Chemical synthesis of N-alkylcarbamate probes

Carbamates consisting of diverse building blocks are described in the literature [51,68]. Different building blocks were selected for the synthesis with the aim to broaden the SAR of the *N*-alkylcarbamate series. Most of the carbamates generated from alkyl alcohols were prepared by activating the alcohol with 1,1′-carbonyldiimidazole in dichloromethane at room temperature followed by coupling to the appropriate amine in dichloromethane at 40 °C (Fig. 1A and B). When amine hydrochlorides were used the reaction was performed in *N*,*N*-dimethylformamide in the presence of triethylamine (Fig. 1E and F). All phenol-based carbamates were synthesized as described in Fig. 1C and D. The phenol was first reacted with p-nitrophenyl chloroformate in the presence of triethylamine in tetrahydrofuran at room temperature. Subsequently, the obtained p-nitrophenyl carbonates were reacted with the amines in tetrahydrofuran at 60 °C. This methodology was also applied for the synthesis of most of the *N*-methyl-2*E*,4*E*-dienamines and for synthesis of two reference compounds first described by Minkkilä et al. [51] (WOBE449 and WOBE491, Fig. 2).