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Characterization of fatty acid amide hydrolase activity by a fluorescence-based assay

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ABSTRACT

Fatty acid amide hydrolase (FAAH) is involved in many human diseases, particularly cancer, pain and inflammation as well as neurological, metabolic and cardiovascular disorders. Therefore, FAAH is an attractive target for the development of low-molecular-weight inhibitors as therapeutics, which requires robust assays that can be used for high-throughput screening (HTS) of compound libraries. Here, we report the development of a fluorometric assay based on FAAH's ability to effectively hydrolyze medium-chain fatty acid amides, introducing *N*-decanoyl-substituted 5-amino-2-methoxypyridine (D-MAP) as new amide substrate. D-MAP is cleaved by FAAH with an 8-fold larger specificity constant than the previously reported octanoyl-analog Oc-MAP (V_{max}/K_m of 1.09 and 0.134 mL min^{-1} mg^{-1}, respectively), with both MAP derivatives possessing superior substrate properties and much increased aqueous solubility compared to the respective *p*-nitroaniline compounds D-pNA and Oc-pNA. The new assay with D-MAP as substrate is highly sensitive using a lower enzyme concentration (1 μg mL^{-1}) than literature-reported fluorimetric FAAH assays. In addition, D-MAP was validated in comparison to the substrate Oc-MAP for the characterization of FAAH inhibitors by means of the reference compounds URB597 and TC-F2 and was shown to be highly suitable for HTS in both kinetic and endpoint assays (*Z′* factors of 0.81 and 0.78, respectively).

Introduction

The ubiquitous membrane-bound enzyme fatty acid amide hydrolase (FAAH, EC 3.5.1.99), a member of the amidase signature family, is abundantly expressed in the CNS as well as in peripheral tissues, such as kidneys, liver, lung, testis, small intestine and prostate [1,2]. The enzyme degrades a variety of physiological active lipids by hydrolysis of amide bonds, including the endocannabinoid anandamide (AEA), oleamide (involved in sleep-induction), as well as *N*-oleoylethanolamide and *N*-palmitoylethanolamide which have appetite-suppressing and anti-inflammatory properties, respectively [1,3–5]. In addition, FAAH was initially thought to hydrolyze the ester bond of a second endocannabinoid, i.e. 2-arachidonyl glycerol, which was concluded from results obtained by recombinant over-expression of the rat liver enzyme in COS-7 cells [6]. However, this result could not be confirmed *in vivo* by using both FAAH knockout mice [7] and treatment with FAAH specific inhibitors, which did not alter the brain level of 2-arachidonyl glycerol [8,9]. FAAH came into focus as target for the development of low-molecular-weight inhibitors due to its involvement in a broad range of human diseases, particularly cancer [10,11], pain and inflammation [12] as well as, among others, neurological [13], metabolic [14,15] and cardiovascular disorders [16]. The recently developed small molecules CAY10401 [10] and URB597 [11,17–19] (Fig. 1) acting as FAAH selective inhibitors have proven to be effective in reducing the proliferative, migratory and invasive potential of various tumor cells. Despite setbacks in the FAAH inhibitor research, such as the tragedy in connection with a French clinical phase I study on BIA10-2474 [20,21] (Fig. 1) (investigating the treatment of “anxiety and motor disorders associated with Parkinson's disease, and chronic pain in people with cancer and other conditions”) as well as the failure of the PF-04457845 [22,23] (Fig. 1) phase II trial on osteoarthritis pain, there is still a strong rationale for the search of potent and selective FAAH inhibitors [24].

The development of such inhibitors often starts from high-throughput screening (HTS) of large compound libraries, with initial HTS hits being the basis for the identification of lead structures. HTS experiments require suitable assays, with methods using chromogenic or fluorogenic substrates being favored over radioassays [25–28]. In

Abbreviations used: DMSO, dimethyl sulfoxide; eq., equivalent; MAP, 5-amino-2-methoxypyridine; MTBE, methyl *tert*-butylether; pNA, *p*-nitroaniline; R_f, retention factor; (v/v), volume solute per volume of total solution; (w/v), weight per volume; (w/w), weight per weight

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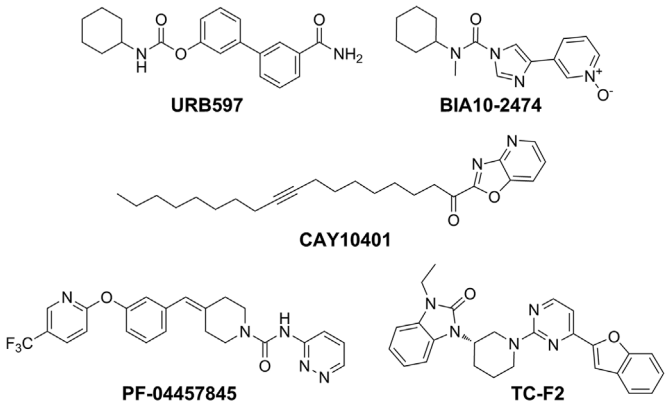

Fig. 1. Molecular structures of reported FAAH inhibitors.

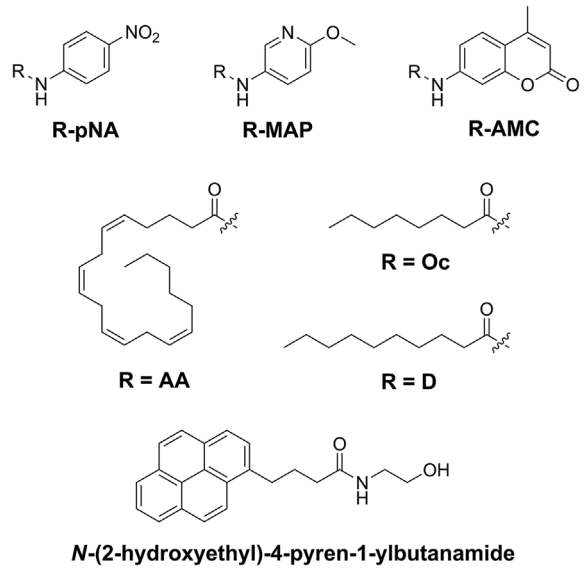

Fig. 2. Molecular structures of literature-known and new FAAH substrates. pNA, *p*-nitroaniline; MAP, 5-amino-2-methoxypyridine; AMC, 7-amino-4-methylcoumarin; AA, arachidonyl; Oc, octanoyl; D, decanoyl. Substrates investigated in this work were *N*-(4-nitrophenyl)octanamide (Oc-pNA), *N*-(4-nitrophenyl)decanamide (D-pNA), *N*-(6-methoxypyridin-3-yl)octanamide (Oc-MAP) and *N*-(6-methoxypyridin-3-yl)decanamide (D-MAP), with the latter one being characterized on FAAH for the first time.

recent years, several spectrophotometric methods have been reported using amides of various fatty acids and colored/fluorescent aromatic amines (Fig. 2), often *p*-nitroaniline (pNA) [29], 7-amino-4-methylcoumarin (AMC) [26–28] and 5-amino-2-methoxypyridine (MAP) [25], respectively. A different concept was applied by Lehr and coworkers [30] who developed a HPLC-based FAAH assay with *N*-(2-hydroxyethyl)-4-pyren-1-ylbutanamide as substrate (Fig. 2) by introducing a fluorescent pyrene moiety into the fatty acid part of the molecule while the ethanolamine part of anandamide remained unchanged.

FAAH substrates initially contained arachidonic acid (AA), which led to potent ligands, such as AA-pNA [29], AA-AMC [26,27] and AA-MAP [25], that suffered, however, from relatively low aqueous solubility and decreased compound stability due to the long (C20) hydrocarbon chain and the presence of potentially labile *cis* double bonds, respectively [25,28]. To circumvent these issues, AA has been exchanged for shorter, saturated alkanoic acids, particularly octanoic acid (Oc) and decanoic acid (D) as in Oc-pNA [29], Oc-MAP [25], D-pNA [29] and D-AMC [28], which also yielded efficient FAAH substrates characterized by high values for k_{cat}/K_m (specificity constant) or V_{max}/K_m.

In this study, we focused on the development of a new fluorogenic amide substrate as the sensitivity of the colorimetric signal arising from chromogenic substrates is limited by a poorer signal to noise ratio and susceptible to interference from colored screening compounds [28]. We preferred MAP to AMC due to the superior sensitivity and water solubility of the former fluorophore, which has recently been shown by Huang *et al.* [25] for Oc-MAP. Since the decanoic acid moiety was found by Patricelli & Cravatt [29] to mediate a higher specificity constant than octanoic acid (as in D-pNA vs. Oc-pNA), we prepared D-MAP for the first time and characterized it in comparison to the literature-known substrates Oc-MAP [25], D-pNA [29], and Oc-pNA [29] with respect to aqueous solubility and substrate properties. In addition, the suitability of D-MAP for inhibition experiments and HTS was investigated by using the inhibitors URB597 and TC-F2 [31,32] (Fig. 1) and by determination of the *Z′* factor, respectively.

Materials and methods

Materials

All reactions were carried out in oven-dried glassware under an argon atmosphere using Schlenk techniques. Solvents were dried by standard methods and distilled prior to use. Nuclear magnetic resonance spectra were recorded on a Bruker Avance 300 MHz. ^{1}H and ^{13}C NMR spectra were processed with MestReNova v.9.0.1 (Mestrelab Research, Santiago de Compostela, Spain). NMR chemical shifts in parts per million (ppm) were referenced to the residual solvent resonance relative to tetramethylsilane (TMS). Signal multiplicities are given as singlet (s), doublet (d), doublet of doublet (dd), triplet (t) and multiplet (m). GC-MS spectra were recorded on a Varian GC-MS 4000 (Varian Inc., Palo Alto, CA, USA) equipped with a J&W scientific DB-5HT column (Agilent Technologies, Santa Clara, CA, USA). Infrared spectra were measured on a Thermo Nicolet 380 FT-IR (Thermo Electron Cooperation, Waltham, MA, USA). IR spectra were processed using OMNIC Specta (Thermo Fisher Scientific, Waltham, MA, USA). Melting points were determined on a melting point apparatus MP 50 (Mettler Toledo, Gießen, Germany). High resolution mass spectra were conducted on a Thermo Fisher Scientific LTQ Orbitrap XL mass spectrometer equipped with a FTMS Analyzer (Waltham, MA, USA). Decanoyl chloride (TCI Europe N.V., Zwijndrecht, Belgium, product number: D0027), *n*-octanoyl chloride (TCI Europe N.V., Zwijndrecht, Belgium, product number: O0039), *p*-nitroaniline (pNA, Acros Organics, product number: 128371000, Thermo Fisher Scientific, Geel, Belgium), 5-amino-2-methoxypyridine (MAP, Acros Organics, product number: 103950250, Thermo Fisher Scientific, Geel, Belgium) and dimethyl sulfoxide (Sigma-Aldrich, product number: 41647, Taufkirchen bei München, Germany) were commercially obtained. Silica gel for chromatography, 0.035–0.070 mm, 60 Å was purchased from Acros Organics (product number: 240360300, Thermo Fisher Scientific, Geel, Belgium). For inhibition experiments on TC-F2 and determination of the *Z′* factor, human recombinant C-terminally His-tagged fatty acid amide hydrolase expressed in *Sf*21 cells was purchased from Cayman Chemical (product number: 10010183, lot numbers: 0481223 and 0516949-1, respectively, Ann Arbor, MI, USA). All other experiments were performed with human recombinant N-terminally His-tagged fatty acid amide hydrolase expressed in *Sf*21 cells, obtained from Cayman Chemical (product number: 10010183, lot number: 0418822-4). The FAAH inhibitor URB597 was purchased from Merck Calbiochem (product number: 341249, Darmstadt, Germany); TC-F2 was commercially obtained from Tocris Bioscience (Bio-Techne, product number: 4355, Wiesbaden, Germany). All reagents were used without prior purification.

Methods

General procedure for the substrate synthesis

To an ice-cold solution of 1 eq. amine and 1.05 eq. triethylamine in 50 mL tetrahydrofuran (abs.), 1.05 eq. acid chloride was added slowly