The fatty-acid amide hydrolase inhibitor URB597 does not affect triacylglycerol hydrolysis in rat tissues

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Abstract

The O-arylcarbamate URB597 (cyclohexylcarbamic acid 3′-carbamoylbiphenyl-3-yl ester; also referred to as KDS-4103) is a potent inhibitor of fatty-acid amide hydrolase (FAAH), an intracellular serine hydrolase responsible for the inactivation of the endogenous cannabinoid anandamide. URB597 demonstrates a remarkable degree of selectivity for FAAH over other serine hydrolases (e.g. cholinesterases) or other components of the endocannabinoid system (e.g. cannabinoid receptors). However, in a proteomic-based selectivity screen based on the displacement of fluorophosphonate-rhodamine (FPR) from mouse brain proteins, it was recently shown that URB597 prevents FPR binding to triacylglycerol hydrolase (TGH) with a median inhibitory concentration of 192 nM. To determine whether this effect correlates with inhibition of TGH activity, we investigated the ability of URB597 to inhibit triolein hydrolysis in rat liver and heart tissues, which are rich in TGH, as well as white adipose tissue (WAT), which is rich in adipose triacylglycerol lipase (TGL) and hormone-sensitive lipase. The results show that URB597 does not affect triolein hydrolysis in any of these tissues at concentrations as high as 10 μM, whereas it inhibits FAAH activity at low nanomolar concentrations. Moreover, intraperitoneal (i.p.) administration of URB597 at doses that maximally inhibit FAAH in vivo (0.3–3 mg kg⁻¹) exerts no effect on triolein hydrolysis and tissue triacylglycerol (TAG) levels in rat liver, heart or WAT. The results indicate that URB597, while potent at inhibiting FAAH, does not affect TGH and TGL activities in rat tissues.

Keywords: URB597; Anandamide; Fatty-acid amide hydrolase; Triacylglycerol hydrolyase

1. Introduction

The intracellular hydrolysis of anandamide (arachidonoyl ethanolamide) [12] to arachidonic acid and ethanolamine is catalyzed by the enzyme fatty-acid amide hydrolase (FAAH), a membrane-bound serine hydrolase that also catalyzes the cleavage of the non-cannabinoid fatty-acid amides oleoylethanolamide and palmitoylethanolamide [2,3,16]. FAAH is expressed at high levels in the central nervous system [6] and mutant mice lacking the gene encoding for this enzyme display a behavioral phenotype that is consistent with an enhanced endocannabinoid-mediated tone [4]. Efforts to develop potent FAAH inhibitors have yielded various families of compounds [14], such as that including the O-arylcarbamate URB597 (cyclohexylcarbamic acid 3′-carbamoylbiphenyl-3-yl ester) [8,13,17]. URB597 inhibits FAAH activity in vitro with median effective concentration (IC₅₀) values of 3 and 5 nM in human and rat brain membranes, respectively [8,15]. Investigations on the actions of URB597 in vivo have revealed that this compound elevates anandamide levels in the brain and exerts marked anxiolytic-like and antidepressant-like effects, which are prevented by the CB₁ cannabinoid receptor antagonist rimonabant [7,8]. The fact that URB597 does not increase brain levels of 2-arachidonoylglycerol, another endocannabinoid ligand, suggests that the pharmacologic effects of this inhibitor are selectively mediated by anandamide [8]. Together, these results...
point to FAAH as an attractive target for the treatment of pain, anxiety and depression.

The selectivity of URB597 for FAAH is supported by in vitro studies, which have shown that this compound has no effect on a variety of serine hydrolase activities, including human and electric-eel acetylcholinesterase, horse plasma butyryl cholinesterase and rat brain monoacylglycerol lipase (MGL) [8]. Furthermore, URB597 does not affect anandamide internalization in human astrocytoma cells [8] and does not interact with a panel of more than 80 receptors, ion channels, and transporters [15]. However, in a proteomic-based selectivity screen based on the displacement of fluorophosphonate-rhodamine (FPR) from mouse brain proteins, Lichtman et al. recently observed that URB597 prevents FPR binding to triacylglycerol hydrolase (TGH) [11]. TGH is a serine hydrolase highly expressed in rat liver and heart tissues, which catalyzes the conversion of triolein and other triacylglycerols (TAG) to free fatty acids and glycerol [5,9]. Although the innovative proteomic approach described by Lichtman et al. offers a high-throughput technique for the rapid screening of compounds against multiple serine hydrolases, it does not provide functional correlates for FPR displacements from proteins, which can only be obtained by direct measurements of enzyme activity. Thus, even though URB597 prevents FPR binding to TGH, it may still be unable to alter the activity of this enzyme. To test this possibility, we examined the effects of URB597 on triolein hydrolysis and TAG levels in rat tissues in vitro and in vivo.

2. Methods

2.1. Animals

Adult male Wistar rats (200–300 g) were housed in standard cages at room temperature on a 12-h light/dark cycle. Water and standard chow pellets were available ad libitum. All procedures met the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

2.2. Chemicals

Anandamide was synthesized in the laboratory by reacting arachidonoyl chloride (Nu-Chek Prep, Elysian, MN) with a 10-fold molar excess of ethanolamine (Sigma–Aldrich, St Louis, MO). Triolein was purchased from Nu-Chek Prep. URB597 was prepared as described [13]. Tetrahydrolipstatin (THL, Orlistat®) was purchased from ChemPacific (Baltimore, MD) and RHC80267 from Biomol (Plymouth Meeting, PA).

2.3. Enzyme preparation

Animals were killed with halothane and tissues were snap-frozen in liquid nitrogen. The samples were homogenized and potterized in ice-cold Tris–HCl (50 mM, 5–9 vol., pH 7.5) containing 0.32 M sucrose. Homogenates were centrifuged at 1000 × g for 10 min at 4 °C. Supernatants were collected and protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). FAAH activity and triolein hydrolysis were measured in the supernatant, as described below.

2.4. FAAH activity

FAAH activity was measured at 37 °C for 30 min in 0.5 mL of Tris buffer (50 mM, pH 7.5) containing fatty acid-free bovine serum albumin (BSA) (0.05%, w/v), protein from tissue homogenates (50 μg from brain and 100 μg from liver and heart), 10 μM anandamide, and anandamide[ethanolamine-3H] (10,000 cpm, specific activity 60 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO). The reactions were stopped with chloroform/methanol (1:1, 1 mL) and radioactivity was measured in the aqueous layers by liquid scintillation counting. For the in vitro experiments the drug was dissolved in dimethyl sulfoxide (DMSO) and added to the reaction without pre-incubation at a final concentration of 1% DMSO.

2.5. Triolein hydrolysis

Triolein hydrolysis was assessed as described by Belfrage and Vaughan [1]. Assays were conducted at 37 °C for 30 min in 0.2 mL potassium phosphate buffer (0.1 M, pH 7.4), protein from tissue homogenates (50 μg from liver, 100 μg from fat, and 300 μg from heart), 850 μM triolein, and triolein[9,10-3H(N)] (750,000 cpm, specific activity 52.6 Ci/mmol; Perkin-Elmer, Wellesley, MA) containing 0.08% Triton X-100 and 0.03% fatty acid-free BSA. The reactions were stopped with heptane/methanol/chloroform (1:1.21:1.25, 3.25 mL) immediately followed by 0.1 M potassium carbonate and boric acid buffer, pH 10.5 (1.05 mL). Radioactivity was measured in the aqueous layers by liquid scintillation counting. For the in vitro experiments the drug was dissolved in DMSO and added to the reaction without pre-incubation at a final concentration of 1% DMSO.

2.6. TAG measurement

Total TAG levels were measured in tissue homogenates using the Infinity TAG kit (Thermo Electron Corporation, Melbourne, Australia).

2.7. Statistical analyses

Results are expressed as the mean ± S.E.M. Statistical significance was evaluated using one-way ANOVA followed by a Dunnett’s post hoc test.

3. Results

3.1. Effects of URB597 on triolein hydrolysis in vitro

URB597 was potent at inhibiting FAAH activity in liver tissue (IC50 = 4.7 nM) (Fig. 1a), but failed to produce significant inhibition of triolein hydrolysis, a measure of TGH activity, at concentrations as high as 10 μM (Fig. 1b). By contrast, the pancreatic lipase inhibitor tetrahydrolipstatin (THL) and the diacylglycerol
3.2. Effects of URB597 on triolein hydrolysis and TAG levels in vivo

Intraperitoneal (i.p.) administration of URB597 (0.3 or 3.0 mg kg$^{-1}$) 1 h prior to sacrifice nearly abolished FAAH activity in rat brain (Fig. 2a) and caused a dose-dependent inhibition of FAAH activity in liver (Fig. 2b) and heart (Table 1). By contrast, URB597 had no effect on triolein hydrolysis and TAG levels in neither liver (Fig. 2c and d) nor heart (Table 1). These results suggest that URB597, when administered in vivo at doses sufficient to abrogate FAAH activity, does not inhibit triolein hydrolysis in two tissues, liver and heart, which are particularly rich in TGH [5,9]. We next tested whether URB597 affects triglyceride lipase activity in WAT, which is enriched in triacylglycerol lipase (TGL) and hormone-sensitive lipase activities [18]. The results show that URB597 did not alter triolein hydrolysis or TAG levels in this tissue (Table 1).

4. Discussion

The O-arylcarmamate URB597 (also referred to as KDS-4103) has been identified as a potent and selective inhibitor of intracellular FAAH activity. URB597 inhibits FAAH activity in human and rat brain membranes with IC$_{50}$ values of 3 and 5 nM, respectively, and blocks brain anandamide hydrolysis in rats following i.p. injections with a half-maximal dose (ID$_{50}$) of 0.15 mg kg$^{-1}$ [8]. This effect appears to be selective in that URB597 does not alter other components of the endocannabinoid system, including cannabinoid receptors and endocannabinoid transport [8]. Furthermore, in vivo inhibition of FAAH by URB597 is accompanied by an elevation of anandamide in the brain and by anxiolytic-like and antidepressant-like effects, which are prevented by the CB$_1$ receptor antagonist rimonabant [7,8].
In vitro studies have shown that URB597 preferentially inhibits FAAH over several other serine hydrolases—including cholinesterases, monoaacylglycerol lipase and cyclooxygenases 1 and 2—and that this compound does not interact with a panel of >80 receptors and ion channels [15]. However, these targets only represent a small fraction of the total proteome. To determine whether URB597 interacts with other proteins, Leung et al. recently introduced a strategy that allows potential inhibitors to be simultaneously counterscreened against many members of the serine hydrolase class of enzymes [10]. The assay determines the interaction of a given compound with serine hydrolases by measuring the compound’s ability to displace an FPR probe directed at the enzyme’s catalytic site [10]. Using this proteomic strategy, Lichtman et al. showed that URB597 prevents FPR binding to two serine hydrolases, FAAH and TGH [11]. However, while this approach yields a broad overview of compound–protein interactions, which could not be obtained by traditional enzyme assay screens, it does not provide a functional correlate between FPR probe displacement and enzyme activity. For this reason, positively screened compounds require secondary testing in activity-based assays to confirm the functional consequence of the FPR displacement. In the present study we conducted such secondary screening. We found that URB597 has no effect on in vitro triolein hydrolysis in rat liver and WAT homogenates at a concentration (10 μM) that is 1000-fold higher than that required to inhibit FAAH activity in rat liver homogenates by ∼90%. We further observed that administration of URB597 at doses that produce maximal FAAH inhibition causes no alteration in triolein hydrolysis or TAG levels in the rat liver, heart, or WAT. Our results suggest that URB597 has no effect on the activities of either TGH, which is highly expressed in rat liver and heart, or TGL, which is predominantly expressed in WAT. Thus, they confirm the target selectivity and potential therapeutic interest of URB597.

References

[1] Belfrage P, Vaughan M. Simple liquid–liquid partition system for isolation of labeled oleic acid from mixtures with glycerides. J Lipid Res 1969;10:341–4.

[2] Calignano A, La Rana G, Giuffrida A, Piomelli D. Control of pain initiation by endogenous cannabinoids. Nature 1998;394:277–81.

[3] Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB. Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature 1996;384:83–7.

[4] Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, et al. Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty-acid amide hydrolase. Proc Natl Acad Sci 2001;98:9371–6.

[5] Dolinsky VW, Sipione S, Lehner R, Vance DE. The cloning and expression of a murine triacylglycerol hydrolase cDNA and the structure of its corresponding gene. Biochim Biophys Acta 2001;1532:162–72.

[6] Freund TF, Katona I, Piomelli D. Role of endogenous cannabinoids in synaptic signaling. Physiol Rev 2003;83:1017–66.

[7] Gobbi G, Bambico FR, Mangieri R, Bortolato M, Campolongo P, Solinas M, et al. Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. Proc Natl Acad Sci 2005;102:18620–5.

[8] Kathuria S, Gaetani S, Fegley D, Valiño F, Duranti A, Tontini A, et al. Modulation of anxiety through blockade of anandamide hydrolysis. Nat Med 2003;9:76–81.

[9] Lehner R, Verger R. Purification and characterization of a porcine liver microsomal triacylglycerol hydrolase. Biochemistry 1997;36:1861–8.

[10] Leung D, Hardouin C, Boger DL, Cravatt BF. Discovering potent and selective reversible inhibitors of enzymes in complex proteomes. Nat Biotechnol 2003;21:687–91.

[11] Lichtman AH, Leung D, Shelton CC, Saghatelian A, Hardouin C, Boger DL, et al. Reversible inhibitors of fatty-acid amide hydrolase that promote analgesia: evidence for an unprecedented combination of potency and selectivity. J Pharmacol Exp Ther 2004;311:441–8.

[12] Mackie K. Cannabinoid receptors as therapeutic targets. Annu Rev Pharmacol Toxicol 2001;414:209–12.

[13] Mor M, Rivara S, Lodola A, Plazzi PV, Tarzia G, Duranti A, et al. Cyclohexylcarbamic acid 3′-esters as fatty-acid amide hydrolase inhibitors: synthesis, quantitative structure–activity relationships, and molecular modeling studies. J Med Chem 2004;47:4998–5008.

[14] Piomelli D. The endocannabinoid system: a drug discovery perspective. Curr Opin Investig Drugs 2005;6:672–9.

[15] Piomelli D, Tarzia G, Duranti A, Tontini A, Mor M, Compton TR, et al. Pharmacological profile of the selective FAAH inhibitor KDS-4103 (URB597). CNS Drug Rev 2006;12:21–38.

[16] Rodriguez de Fonseca F, Navarro M, Gomez R, Escuredo L, Nava F, Fu J, et al. An anorexic lipid mediator regulated by feeding. Nature 2004;430:453–7.

[17] Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, et al. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. Science 2004;306:1383–6.

Table 1
Effects of URB597 on FAAH activity, triolein hydrolysis and total TAG levels in rat heart and white adipose tissue

|                     | Heart                         | White adipose tissue |
|---------------------|-------------------------------|----------------------|
|                     | Vehicle                        | 0.3 mg kg⁻¹    | 3.0 mg kg⁻¹    | Vehicle                        | 0.3 mg kg⁻¹    | 3.0 mg kg⁻¹    |
| FAAH activity (pmol/min/mg protein) | 32.5 ± 2.3                    | 10.7 ± 1.5**       | 11.7 ± 2.0**   | ND                            | ND                | ND                |
| Triolein hydrolysis (pmol/min/mg protein) | 105.0 ± 0.7                   | 11.7 ± 0.8         | 11.8 ± 1.3     | ND                            | ND                | ND                |
| Triglycerides (µg/mg protein) | 450.8 ± 17.4                   | 473 ± 36.9         | 461.6 ± 19.5   | 3815 ± 143                    | 3389 ± 411        | 3560 ± 80.0      |

Data are expressed as the mean ± S.E.M. for each parameter measured 1 h following injection of vehicle, 0.3 or 3.0 mg kg⁻¹ URB597. **P < 0.01 vs. vehicle-treated animals; ANOVA with Dunnett’s post hoc test; vehicle and 3.0 mg kg⁻¹ groups, n = 6; 0.3 mg kg⁻¹ group, n = 4. ND, not determined.