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Journal
Pharmacological Research, 54(5)

ISSN
1043-6618

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Publication Date
2006-11-01

DOI
10.1016/j.phrs.2006.06.008

ELSEVIER

Pharmacological Research 54 (2006) 341–344

Pharmacological research

www.elsevier.com/locate/yphrs

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

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Accepted 7 June 2006

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 hydrolase

1. Introduction

The intracellular hydrolysis of anandamide (arachidonoylethanolamide) [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 (IC_{50}) 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 CB_1 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

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1043-6618/$ – see front matter © 2006 Published by Elsevier Ltd.
doi:10.1016/j.phrs.2006.06.008

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 $1000 \times g$ 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-^{3}H] (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-^{3}H(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 (IC_{50} = 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

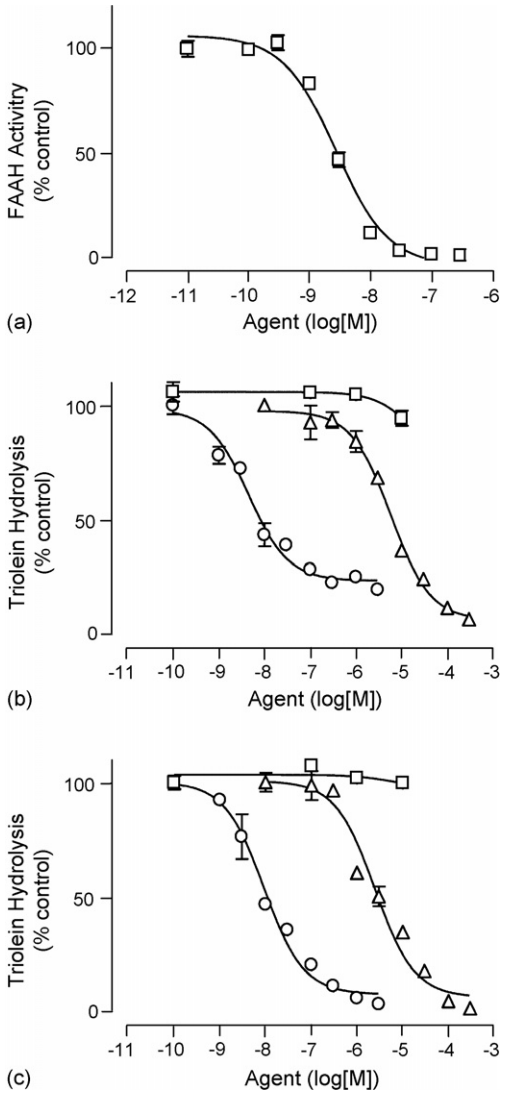

Fig. 1. Effects of URB597 on FAAH activity and triolein hydrolysis *in vitro*. (a) URB597 (squares) inhibits FAAH activity in liver homogenates (IC_{50} = 4.7 nM). (b) URB597 has no significant effect on triolein hydrolysis in liver at concentrations up to 10 μM. By contrast, THL (circles) and RHC80267 (triangles) inhibit triolein hydrolysis (IC_{50} = 3.4 nM and *5.8 μM, respectively). (c) URB597 has no effect on triolein hydrolysis in white adipose tissue homogenates; THL and RHC80267 both inhibit triolein hydrolysis (IC_{50} = 8.8 nM and 1.7 μM, respectively). Results are the mean of two independent experiments performed in triplicate. *Results are the mean of one experiment performed in triplicate.

lipase inhibitor RHC80267 inhibited triolein hydrolysis in liver with IC_{50} of 3.4 nM and 5.8 μM, respectively (Fig. 1b). In addition, URB597 exerted no inhibitory effect on triolein hydrolysis in rat WAT, where THL and RHC80267 were markedly effective (IC_{50} = 8.8 nM and IC_{50} = 1.7 μM, respectively) (Fig. 1c). These results indicate that URB597 does not inhibit the activities of major lipolytic enzymes in broken-cell preparations of rat liver and white adipose tissue.

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 affect 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).

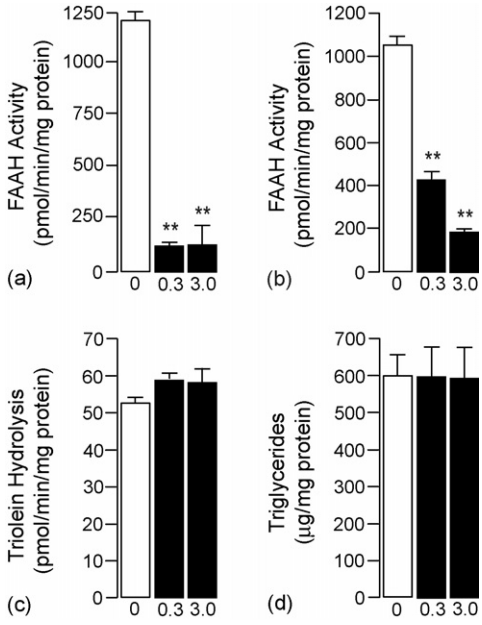

Fig. 2. Effects of URB597 on FAAH activity, triolein hydrolysis activity and total TAG levels in brain and liver tissues. One hour after i.p. administration, URB597 (0.3 and 3.0 mg kg^{-1}) (a) nearly abolishes FAAH activity in brain tissue and (b) dose dependently reduces FAAH activity in liver tissue. By contrast, URB597 does not affect (c) triolein hydrolysis or (d) total TAG levels in liver tissue at any of the doses tested. V, vehicle; $^{**}P < 0.01$ 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, $n = 4$.

4. Discussion

The *O*-arylcarbamate 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].

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)	10.5 ± 0.7	11.7 ± 0.8	11.8 ± 1.3	161.2 ± 13.0	175 ± 21.3	175.8 ± 9.1
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.

In vitro studies have shown that URB597 preferentially inhibits FAAH over several other serine hydrolases – including cholinesterases, monoacylglycerol 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–proteome 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.

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