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# Characterization of [3-(3-carbamoylphenyl) phenyl] N-cyclohexyl carbamate, an inhibitor of FAAH: effect on rat liver FAAH and HEK293T-FAAH-2 deamination of oleamide, arachidonamide and stearoylamide

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## ABSTRACT

The carbamate, [3-(3-carbamoylphenyl) phenyl] N-cyclohexyl carbamate (URB597) is a known irreversible inhibitor of fatty acid amide hydrolase (FAAH) which is capable of increasing intracellular endocannabinoid levels to measurable antidepressant and analgesic effects in animal models. Several endocannabinoid-like compounds such as the primary fatty acid amides (PFAMs) are also important signaling molecules which control many physiological processes and exhibit the characteristic cannabimimetics though the physiological functions of some remain elusive. Thus, PFAMs remain candidates for examination. Since the initial discovery of URB597, little has been reported on its ability to inhibit the FAAH-dependent metabolism of important PFAM substrates. Also, the mode of inhibition of FAAH hydrolysis of substrates by URB597 has not been characterized. FAAH-2 was expressed in HEK293T cells whereas FAAH was assayed from rat liver preparations. Activity assay employed was a modification of a high throughput fluorimetric screening method[1].

URB597 exhibited concentration-dependent inhibition of both FAAH and FAAH-2 hydrolysis of the 3 PFAMs. The potency ($pIC_{50}$) of URB597 obtained against FAAH activity were 6.6 ± 0.1, 6.0 ± 0.1 and 6.0 ± 0.1 for ODA, ArDA and SyDA. URB597 also inhibited FAAH-2 metabolism of PFAMs in a rank order of ODA > ArDA ≈ SyDA (respective $pIC_{50}$ values were 6.90 ± 0.10, 6.77 ± 0.02 and 6.75 ± 0.10). The respective $K_m$ and $V_{max}$ values for FAAH were 43.0 ± 6.0 μM and 14 ± 2 $nmol.min^{-1}.(mg\ protein)^{-1}$ for ArDA and 60 ± 4 μM and 14 ± 1 $nmol.min^{-1}.(mg\ protein)^{-1}$ for ODA. Our results suggest that URB597 inhibited FAAH hydrolysis of both ArDA and ODA in an uncompetitive manner.

**Key words:** Arachidonamide, FAAH, FAAH-2, Hydrolysis, Inhibition, Mode, Oleamide, Potency, Stearoylamide, URB597.

## Introduction

$\Delta^9$-Tetrahydrocannabinol (THC) and other cannabinoid agonists have long been recognized to possess medicinal properties. THC is reported to provide relief to chronic pain sufferers (e.g. in cancer, arthritis, multiple sclerosis, AIDS), to stimulate appetite or weight gain, to suppress growth of tumours by increasing cell apoptosis and to ease anxiety[2,3]. THC also possesses anti-inflammatory, anti-emetic and anti-convulsive properties, among others, thus, making this cannabinoid a very promising drug for therapeutic purposes[4]. These agents however, also produce undesirable side effects which limit their utility as therapeutic agents. These include impairments in cognition and motor control, altered senses, reduced coordination and balance, panic, hallucinations and blurred vision. The difficulty, therefore, remains how to exploit the therapeutic benefits of cannabis and yet limit the harmful side effects.

One attractive approach to maintain the beneficial effects of cannabinoid receptor activation, while circumventing adverse psychoactive effects is to

elevate endocannabinoid tone by inhibiting endocannabinoid degradation enzymes. A number of FAAH inhibitors have been described to date and have been well reviewed[5]. These include substrate analogues such as oleyltrifluoromethylketone (OTMK)[6], arachidonoyl serotonin (AA-5HT)[7], arachidonoyl trifluoromethyl ketone (ATMK)[8] and diazomethylarachidonyl ketone (DAK)[9]. Others include serine protease inhibitors (phenylsulphonyl fluoride; PMSF)[10], an arachidonoyl binding site directed phosphonylation reagent, methoxy arachidonoyl fluorophosphonate (MAFP)[11] and α-ketoheterocycles[8] as well as the *o*-aryl carbamates[12,13]. The first carbamate inhibitors of FAAH were designed by modifying the structure of a known inhibitor of the serine hydrolase acetylcholinesterase. Characterization of the structure–activity profiles for *o*-aryl carbamate compounds showed that introduction of small polar groups in the *meta* and *para* positions of the distal phenyl ring of URB524 ($IC_{50}$ = 63 nM), greatly improved inhibitory potency[13]. Most potent in the series was the *m*-carbamoyl derivative URB597 (Fig 1; $IC_{50}$ = 4.6 nM) a relatively selective, irreversible inhibitor of FAAH[13].

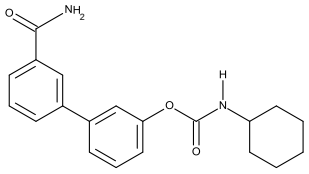


**Fig 1: Stucture of URB597**

FAAH is an integral membrane enzyme of the serine family of hydrolases and is the primary enzyme responsible for the degradation of the endocannabinoid anandamide (AEA) and related fatty acid amides. FAAH is thus, a modulator of endocannabinoid and fatty acid ethanolamide signaling and may be of therapeutic significance. Inhibition of FAAH therefore results in the accumulation of AEA in the central nervous system and periphery where it activates cannabinoid receptors. Cannabinoid ligands possess anti-inflammatory, antinociceptive, analgesic and immunosuppressive properties among others, hence, inhibitors of AEA and related fatty acid amide degrading enzymes (e.g. FAAH, FAAH-2, NAAA, MAGL, COX-2, LOX) may be of therapeutic value[14] via augmentation of endocannabinoid signaling *in vivo*.

The pharmacological properties of URB597 as a highly potent and selective inhibitor of FAAH leading to elevated levels of AEA and other *N*-acyl ethanolamides (NAEs) *in vivo* has been well documented. URB597 produces profound inhibition of FAAH in mice and rats which display elevated brain levels of these lipid amides coupled with highly reduced sensitivity to pain[15,16,17]. URB597 also exerts anxiolytic-like[17], antidepressant-like[18] and antihypertensive[19] effects in rodents which support FAAH as a potential therapeutic target. $FAAH^{-/-}$ mice are reported to possess highly elevated levels of fatty acid amides in the central nervous system and some peripheral tissues, a metabolic phenotype which correlates with a $CB_1$-dependent reduction in pain sensation in these animals. $FAAH^{-/-}$ mice also exhibit a reduced inflammation in multiple peripheral models[20].

FAAH inhibitors designed to date possess distinct sets of attributes and deficiencies that reflect some of the most challenging aspects of FAAH inhibitor development. One vital challenge for reversible inhibitors is that, whereby inhibition of FAAH leads to elevated levels of NAE substrates which indirectly reduce the potency of a given inhibitor by mass-action competition with the substrates[21], URB597 is a selective FAAH inhibitor and interacts neither with substrate transport nor $CB_1$ or $CB_2$ receptors. Irreversible inhibitors (e.g. URB597) produce a more complete blockade of FAAH *in vivo*. URB597 unfortunately also inhibits other serine hydrolases, of which there are over 200 members in the periphery, inhibitor selectivity is thus a major challenge[20,22].

The PFAMs of which oleamide (ODA) is the most explored, are also important signaling molecules in the mammalian nervous system. They bind to many drug targets and demonstrate control over sleep, locomotion, angiogenesis and many other physiological processes[23]. ODA is a full agonist at $CB_1$ and exhibits the characteristic *in vivo* analgesic and cannabimimetics of AEA in mice and induces sleep in a manner that is indistinguishable from physiological sleep, albeit without cannabinoid receptor binding, however, consistent with those required of serotonergic and GABAergic neurotransmission[24]. Thus, it has been suggested that

ODA has the potential for being developed as a sleep aid that lacks the side effects of sedatives, hypnotics and the suicide-abuse potential of central nervous system depressants. Palmitamide is reported to possess weak anticonvulsant properties[25] while arachidonamide (ArDA) affects gap junction communication[9].

More recently, additional PFAMs including stearoylamide (SyDA) and myristamide have been isolated from human tear gland secretions however, their action remain elusive[26]. The fact that ArDA for example, remains the best substrate of FAAH suggests that it represents a key signaling molecule in this category. Thus, PFAMs remain candidates for examination as existing and/or new targets for FAAH and should be analyzed in the presence of FAAH inhibitors to block their rapid degradation[27].

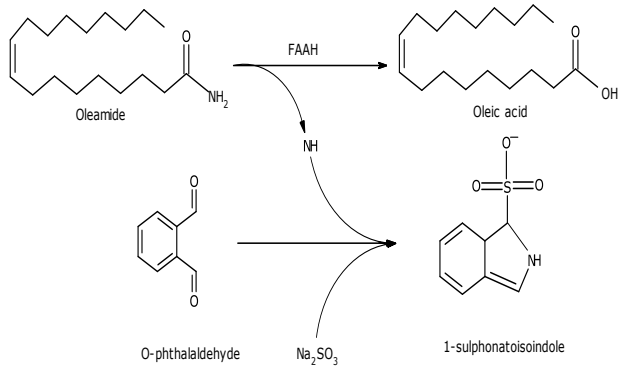


**Fig 2: Mechanism of fluorescence generation.** The activity of FAAH is quantified as the amount of ammonia released after FAAH catabolism of primary amide substrates. The figure illustrates formation of 1-sulphonatoisoindole a highly fluorescent compound from ammonia generated via FAAH hydrolysis of oleamide. Ammonia released in the presence of o-phthalaldehyde and sodium sulphite forms 1-sulphonatoisoindole whose fluorescence is determined to represent a measure of FAAH activity.

Since the initial discovery of URB597, little has been reported on the characteristic *in vitro* inhibitory action of URB597 on rat FAAH and human FAAH-2 dependent metabolism of important PFAMs. The mode of inhibition of these FAAH substrates by URB597 has not been characterized. The mechanism of FAAH inhibition by URB597 remains unclear, thus, complicating efforts to rationally design agents with superior efficacy[27].

Here we confirm URB597 as a highly potent inhibitor of rat liver FAAH and HEK293T-FAAH-2 (HEK293T cells previously transfected with FAAH-2 expression plasmid) deactivation of 3 different PFAMs with a mode of inhibition that is uncompetitive in nature. We employed a high throughput screening assay method based on detection of ammonia generated from FAAH hydrolysis of primary amides by trapping with sulphite and OPA to generate a stable fluorescent isoindole derivative which is quantified by fluorescence spectroscopy (Fig 2)[1]. The clinical significance of the selected PFAMs has also been discussed.

## Materials and Methods

### Preparation of rat liver homogenate

Wistar rat liver stored at -40 °C was thawed, weighed and homogenized in 0.2 M Potassium Phosphate buffer (P. buffer) pH 7.4 with an ultraturrax in a volume of 6.1 ml/g wet weight. The mixture was centrifuged twice at 250 g for 10 min at 4 °C. The resulting supernatants were combined and centrifuged twice at 20,000 g for 30 min at 4 °C after which the membrane containing pellet was resuspended at 1:1$^{w}/_{v}$ in P. buffer pH 7.4 and stored at -40 °C in 1 ml aliquots until required for protein and enzyme assays.

### Preparation of HEK293T-FAAH-2 cell lysate

DH5α *E. coli* competent cells were transformed with FAAH-2-pcDNA3.1 construct and incubated at 37 °C overnight after which the plasmid was purified with the QIAGEN® Maxiprep kit following manufacturer's instructions (QIAGEN Plasmid Purification Handbook, 2003). The amount of nucleic acid in each purified sample was determined with the NanoDrop 2000 reader (Thermo Scientific, Wilmington, USA) and visualized by 1.0 % agarose gel electrophoresis on a UV Trans-illuminator. HEK293T cells were transiently transfected with the FAAH-2 expression plasmid using the polyethylenimine (PEI) transfection protocol from Cold Spring Harbour Laboratories[28,29]. Cells were normally seeded 1:7; in 20 ml complete culture medium (500 ml DMEM, 10 % FBS, 5 % L-glutamine) for ~48 h in 75 $cm^2$ flasks to ~70 % confluence before transfection. Cell count at seeding was normally between 69 and 96 % live cells. HEK293T-FAAH-2 cells were harvested by creating cell suspensions using cell dissociation buffer and pelleted at 2,000 g for 10 min at 4 ˚C and stored at -80 ˚C until required for enzyme assays.

### Potency of URB597 to FAAH and FAAH-2 hydrolysis of PFAMs

### FAAH activity assay:

FAAH activity from rat liver was assessed as previously described[1], using an ammonia detection methodology (Fig 2) and primary amide substrates; 100 μM ODA, 50 μM ArDA and 10 μM SyDA (synthesized by Dr. Stephen Alexander, School of Life Sciences, University of Nottingham). Rat liver homogenate from above was pre-incubated with concentrations of URB597 (Cayman Chemical Company, Michigan, USA) at 37 °C for 30 min in 96-well plates (Thermo Scientific Inc., Waltham, USA) with shaking (50 x 10 rpm) prior to substrate addition to a 100 μl total assay volume and incubation at 37 °C for 30 min. Ammonium sulphate standard concentrations (0.0 - 0.1 mM) were added to separate wells after which all reaction mixtures were halted with an equivalent assay volume of o-phthalaldehyde (OPA) (Pierce Ltd, Illinois, USA) developing solution (0.4 M P. buffer, pH 11.5) and incubated at room temperature for 15 min before assessing fluorescence using FLUOstar Galaxy (BMG LABTECH GmbH, Ortenberg, Germany) (ex 390, em 450 nm).

**HEK293T-FAAH-2 activity assay**

Cells from each 75 $cm^2$ flask were resuspended in P. buffer pH 7.4, homogenized with ultraturrax and diluted with P. buffer pH 7.4 for activity assays. To determine the expression of a functional FAAH-2 enzyme, protein concentration dependence of ODA hydrolase activity of HEK293T cells transfected or mock transfected with FAAH-2 expression plasmid were assessed as above using rat liver FAAH as positive control.

**Mode of inhibition of rat liver FAAH deamination of ODA and ArDA by URB597**

FAAH activity from rat liver was assessed in the presence of 50 nM URB597 as above using a range of substrate concentrations, 6.25 – 400 μM ODA and 1.25 – 80 μM ArDA. The protein content of each tissue was determined by the Lowry protocol[30] using standard concentrations (0 - 300 μg/ml) of serum bovine albumin. Protein content of tissues was interpolated from the standard Lowry curve.

**Statistical analysis**

Data obtained was entered into a Microsoft excel 2010 spread sheet and analyzed with GraphPad Prism computer software programme (GraphPad Software Inc., San Diego, CA USA) version 6.0. For the concentration-inhibition curves (Fig 4 and 5), specific activity obtained at each inhibitor concentration were normalized and analyzed using the inbuilt log (inhibitor) verses response variable slope (robust fit) constrained (Bottom/residual activity = 0.0 %) and plotted as percentage of control. Values for $pIC_{50}$ were then expressed as equal to negative $logIC_{50}$ of triplicate assessments of the corresponding software interpolated $logIC_{50}$ values. The amount of ammonia generated via PFAM metabolism by FAAH was interpolated from Michealis-Menten standard ammonium sulphate curve using the inbuilt non-linear Michealis-Menten enzyme kinetics. The $V_{max}$ and $K_m$ values were then computed with the Michealis-Menten enzyme kinetics using the protein concentrations of the preparations obtained from the Lowry protocol.

**Results**

Agarose gel electrophoresis of purified FAAH-2-pcDNA3.1 obtained above is shown in Fig 3 with expected size of 7,072 bp.

URB597 caused a concentration-dependent inhibition of FAAH activity from rat liver with high potency ($pIC_{50}$ = 6.6 ± 0.1) when 100 μM ODA was used as substrate (Fig 4a). URB597 was more potent as an inhibitor of FAAH-2 ODA hydrolysis with a $pIC_{50}$ of 6.9 ± 0.1 (Fig 4b). When ArDA and SyDA were used, there was no significant difference between the potencies against FAAH activity. In the same way, URB597 inhibited FAAH-2 metabolism of ArDA and SyDA with approximately the same potency (Fig 5; Table 1). URB597 was however, more potent as an inhibitor of FAAH-2 ArDA and SyDA hydrolase activity than for rat liver FAAH activity. URB597 inhibited ODA deamination for both isozymes PFAM hydrolysis with a rank order of ODA > ArDA ≈ SyDA.

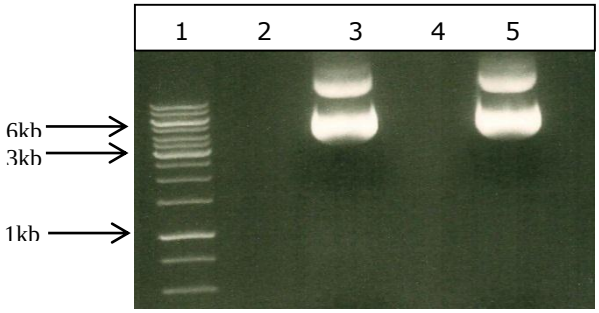


**Fig 3: Agarose gel electrophoresis of purified FAAH-2-pcDNA3.1.** Lane 1 shows the molecular weight marker (O'GeneRuler) while 3 and 5 show purified FAAH-2-pcDNA3.1 from 2 different cultures. Lanes 2 and 4 did not contain any samples.

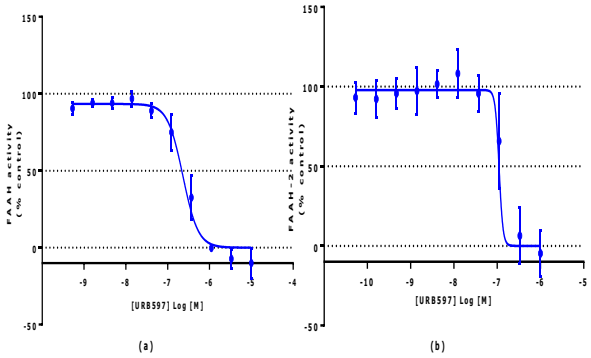


**Fig 4: Concentration-dependence of (a) rat liver FAAH and (b) HEK293T-FAAH-2 ODA hydrolase activity inhibition.**
Data are mean ± SEM of triplicate assessments conducted on four different preparations.

In pilot experiments, the affinity ($K_m$) of ODA, ArDA and SyDA at rat liver FAAH and HEK293T-FAAH-2 were determined, therefore a range of concentrations of URB597 was assessed as inhibitor of rat liver FAAH or HEK293T-FAAH-2 hydrolysis of the three PFAMs, assayed at, close to or greater than the $K_m$ value[1,31,32].

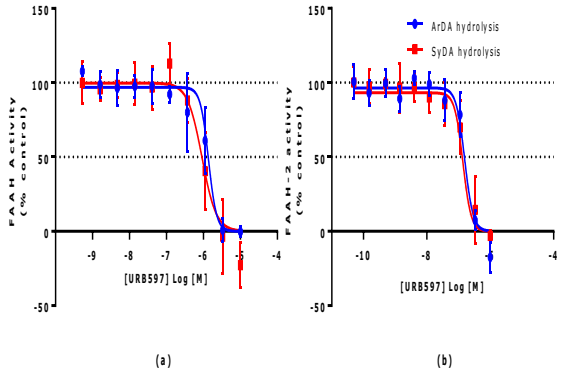


**Fig 5: Concentration-dependence of (a) rat liver FAAH and (b) HEK293T-FAAH-2 hydrolase activity inhibition.**
Data are mean ± SEM of triplicate assessments conducted on four transient transfects or rat liver preparations.

**Table 1: Potency of URB597 to FAAH and FAAH-2 substrate deamination**

| Tissue | Substrate | Substrate concentration/µM | †$pIC_{50}$ |
|---|---|---|---|
| FAAH-2 | ODA | 100 | 6.90 ± 0.10 |
| | ArDA | 50 | 6.77 ± 0.02 |
| | SyDA | 50 | 6.75 ± 0.10 |
| FAAH | ODA | 100 | 6.60 ± 0. 10 |
| | ArDA | 50 | 6.00 ± 0.10 |
| | SyDA | 10 | 6.00 ± 0.10 |

† presented as Mean ± range of triplicate assessments conducted on four different transfects or rat liver preparations.

## Mode of inhibition of rat liver FAAH substrate hydrolysis by URB597

At 50 nM ([URB597] log [M] = -7.3), URB597 produced a slight inhibition of indicated substrate hydrolysis by both FAAH and FAAH-2, hence the mode of inhibition was investigated at 50 nM URB597. Michealis-Menten analysis revealed a reduction of $V_{max}$ for both ODA and ArDA hydrolysis by rat liver FAAH with significant reduction in substrate affinity (Table 2). This is indicative of uncompetitive mode of inhibition of FAAH activity.

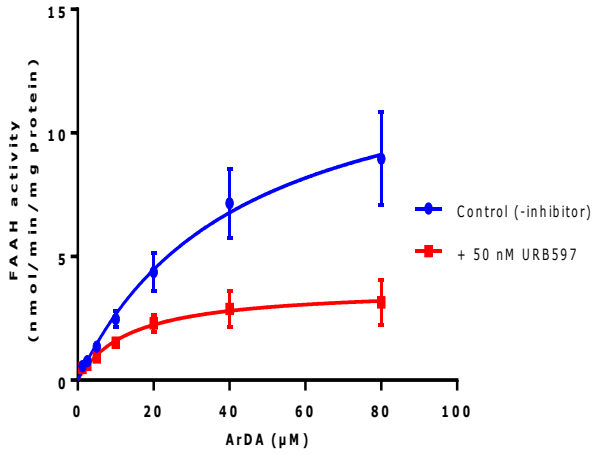


**Fig 6: Mode of inhibition of rat liver FAAH hydrolysis of ArDA by URB597.**
Data are mean ± SEM of triplicate assessments conducted on four different preparations.

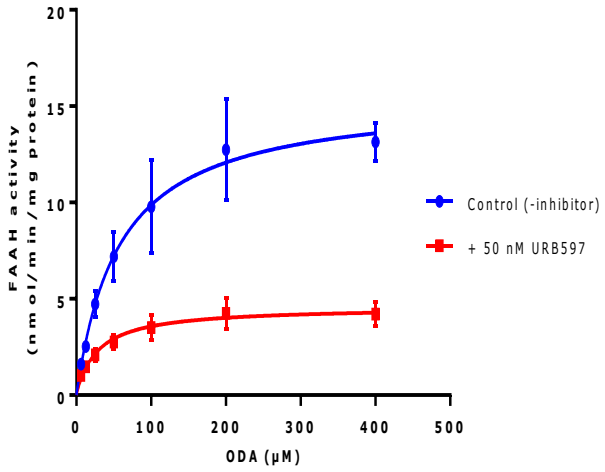


**Fig 7: Mode of inhibition of rat liver FAAH hydrolysis of ODA by URB597.**
Data are mean ± SEM of triplicate determinations conducted on four preparations.

**Table 2: The mode of inhibition of rat liver FAAH ODA and ArDA hydrolysis by URB597**

| | Substrate hydrolysis | | Substrate |
|---|---|---|---|
| | $K_m$ (μM) | $V_{max}$ (nmol/min/mg protein) | |
| **Control** | 60 ± 4.0 | 14 ± 1.0 | ODA |
| **+ 50 nM URB597** | 28 ± 2.0 | 5 ± 0.4 | |
| **Control** | 43.0 ± 6.0 | 14.0 ± 2.0 | ArDA |
| **+ 50 nM URB597** | 14.0 ± 3.0 | 4.0 ± 0.7 | |

Data are mean ± SEM of triplicate assessments conducted on four different preparations.

## Discussion

Most *in vitro* and *in vivo* inhibition studies involving FAAH or FAAH-2 activity have focused on the use of radiolabelled [$^3$H]-AEA as substrate[33]. Our investigation was therefore designed to utilize PFAMs, ODA (C18:1), ArDA (C20:4) and SyDA (C18:1 triple bond).

### Pharmacological properties of URB597 *in vitro*

A simple and inexpensive high throughput fluorimetric screening assay which eliminates the use of chromatography and hazardous radiochemicals and solvents such as chloroform, was employed to characterize the effect of URB597 on FAAH activity from rat liver and that of FAAH-2 expressed in HEK293T cells. FAAH metabolizes PFAMs to the corresponding fatty acids. Ammonia released in the presence of sodium sulphite reacts with OPA to form 1-sulphonatoisoindole which is detected by fluorescence spectrometry (Fig 2).

The $IC_{50}$ values obtained reveal that URB597 is highly potent against both FAAH ($IC_{50}$ for ODA hydrolysis = 251 nM, ArDA = 1 μM, SyDA = 1 μM) and human recombinant FAAH-2 ($IC_{50}$ for ODA hydrolysis = 125 nM), ArDA (170 nM) and SyDA (179 nM). URB597 was therefore more potent against FAAH-2 than FAAH metabolism of all 3 PFAMs. These findings are consistent with earlier reports that URB597 and OL-135 show greater potency for FAAH-2 compared with FAAH[17,20]. Similarly studies have also observed a higher potency of URB597 for human recombinant FAAH-2 ODA hydrolase activity than for human recombinant FAAH[34], with JP104, an agent that combines structural features of URB597 and α-keto heterocycles being the most potent[12,13,17,20].

### Mechanism of inhibition of FAAH and FAAH-2 hydrolysis of PFAMs by URB597

URB597 was rationally designed on the basis of carbamate-containing serine protease inhibitors and was co-crystallized with humanized FAAH[13,33]. Carbamates such as URB597 are proposed to inhibit FAAH by an irreversible, covalent mechanism involving carbamylation of Ser241[12,17]. This hypothesis is yet to be directly confirmed. In contrast to earlier reports, other investigations showed that URB597 displayed a slowly reversible inhibitory mechanism of FAAH[5].

The proposed model of URB524 and URB597 binding in the FAAH active site suggests that their biphenyl substituents mimic the arachidonoyl chain of AEA and reside in the acyl chain binding channel of FAAH[13,34]. However, in this model, the orientation of binding of FAAH would place the *N*-alkyl substituents of carbamates in the cytoplasmic access channel of FAAH in positions that are more suitable for leaving groups (similar to that of AEA). This could possibly result from carbamylation of the enzyme's nucleophile[12]. Chemical reactivity studies have also questioned this model since the extent of electrospray ionization-induced cleavage of the C(O)-O bond in a series of FAAH carbamate inhibitors was found to correlate with their potency of inhibition suggesting that O-aryl esters may instead serve as leaving groups in FAAH carbamate reactions[34,35]. To ascertain the mechanism of carbamate inhibition of FAAH therefore, purified recombinant enzyme was treated with URB532 and URB579 for 1 h after which the protein samples were subjected to tryptic digestion and MALDI-TOF mass spectrometry analysis[12]. No evidence of C(O) – aryl modification of FAAH was observed in the URB532 nor the URB597 reaction. Liquid chromatography-tendam electrospray analysis however, suggested Ser241 as the site of carbamylation[12].

Our data (Fig 6 and 7; Table 2) suggest that FAAH inhibition by URB597 is uncompetitive in nature and is carbamylated to Ser241 which is the nucleophile proposed to interact with FAAH substrates[36,37,38]. This is supported by the observation that there is a significant difference between the $K_m$ values; $K_m$ values for reactions originally incubated with URB597 were significantly much less than controls (Table 2). $V_{max}$ values were also lower than controls. It is predicted that Lineweaver-Burk plots will have

different 1/K (x-intercept) and $1/V_{max}$ (y-intercept). The $K_m$ for ODA hydrolysis by FAAH was ~1.4x higher than that for ArDA. This suggests that, the affinity of FAAH is higher for ArDA compared with ODA.

**Clinical relevance of selected PFAM substrates**

First discovered in the cerebrospinal fluid of sleep-deprived cats which makes it a potential agent to be developed as sleep aids, ODA now a known endogenous sleep-inducing lipid hormone also has a range of actions that could give it considerable modulatory power[9,39,40]. ODA interacts with voltage-gated $Na^+$ channels and allosterically with $GABA_A$ and 5-HT7 receptors, and is a direct agonist at TRPV1 and $CB_1$ receptors[24,41,42,43,44]. ODA therefore exhibits the characteristic cannabimimetic effects, that is, the characteristic "tetrad" of effects evoked by cannabinoid receptor agonists *in vivo*, including hypothermia, hypoactivity, analgesia and catalepsy[39,45,46]. ODA has also been demonstrated to be involved in the inhibition of gap-junctional communication and therefore might play a significant role in myocardial development[47,48,49]. One most potent effect of ODA is its ability to cause vasodilation. This has been demonstrated in rat small mesenteric artery apparently via activation of another pertussis toxin-sensitive, G protein–coupled receptor yet to be identified, rather than $CB_1$ receptor[50]. These properties indicate ODA is a reliable candidate for the development of analgesics and drugs for the treatment of sleep disorders among others.

ODA and its derivatives are efficient inhibitors of connexin functions[51,52]. Daily intraperitoneal injections of ODA derivatives named MI-18 and MI-22, potently inhibit connexin 26 enhanced spontaneous metastasis of mouse BL6 melanoma cells[53]. This suggests that MI-18 and MI-22 ODA derivatives may serve as prototypes for novel and clinically important anticancer drugs for cancer treatment. This notwithstanding, ODA increases appetite and therefore increases food intake, another known feature of cannabinoid pharmacology and was demonstrated by intraperitoneal injections of ODA which enhanced food intake for up to 3 h[14,54]. ODA possesses seizure limiting properties and might represent an endogenous anticonvulsant as well as an important regulator of arousal[55]. ODA also possesses antianxiety effect which suggests that it is a candidate for development of antianxiety drugs[56].

There is limited literature on ArDA and SyDA so far. ArDA is a weak $CB_1$ and $CB_2$ agonist and acts as a potent inhibitor of gap junction cell-cell communication[9,52]. ArDA is a competitive AEA $Na^+$-independent transporter inhibitor. N-(4-hydroxyphenyl)-arachidonamide selectively inhibits AEA transport and attenuates reinstatement of nicotine-seeking behaviour *in vivo* and prevents development of nicotine-induced conditioned place preference[57,58,59]. These findings suggest that AEA transport inhibition may serve as a new target for development of medications for treatment of tobacco dependence[60]. Since N-(4-hydroxyphenyl)-arachidonamide does not closely mimic the spectrum of pharmacological responses produced by direct cannabinoid agonists, it can be administered to selectively increase endogenous brain levels of AEA without altering levels of OEA or PEA[61,62,63].

Since it's isolated, SyDA's physiological significance has remained elusive[26]. However, the novel finding of SyDA and other fatty acid amides in tear gland and meibomian gland secretions might result in additional insights into the role (e.g. protection of ocular surface from dehydration and therefore against dry eyes[64,65]) of this family of lipids in human biological systems with novel therapeutics.

**Target selectivity of URB597**

There remains a strong skeptism regarding the use of irreversible inhibitors as clinical drug candidates. Yet, approximately 30 % of drugs commercially available inhibit target enzymes via a covalent irreversible mechanism. This is due to high inherent reactivity of functional groups generally associated with covalent protein modifiers. URB597 has demonstrated high selectivity against several serine hydrolases that have been screened in parallel. URB597 for instance, was highly selective as an inhibitor of FAAH but also evoked significant inhibitory action on triacylglycerol hydrolase ($IC_{50}$, 192 nM)[15,17,20]. These studies revealed that several serine enzymes such as triacylglycerol hydrolase and lipoprotein lipase, which should not be targets of FAAH inhibitors, are affected by carbamate inhibitors of FAAH. Triacylglycerol lipase and lipoprotein lipase regulate the mobilization of triacylglycerides in the periphery, hence, their inhibition may produce untoward effects on the ratio

of high-density and low density lipoprotein. Further studies are therefore required in this aspect to ascertain the mechanism of URB597 inhibition of FAAH or novel compounds that will exhibit better inhibitory potential, higher selectivity and safety profiles.

**Plasma concentrations and potential of URB597 as a therapeutic**

URB597 is reported to be absorbed after oral administration[66]. Maximum plasma levels of URB597 measured 15 min after 10 and 50 mg/kg oral dosing of mice were 47 nM and 266 nM respectively. The drug reportedly cleared from the circulation within 1 h after 10 mg/kg dose and 12 h after 50 mg/kg dose. Our data therefore suggests that a dose of URB597 > 50 mg/kg (which will evoke plasma concentrations > the obtained $IC_{50}$ values for the PFAMs in our findings) will be required to inhibit the degradation of the PFAMs by FAAH and FAAH-2. Pharmacokinetic experimental results indicate that URB597 is absorbed at a moderate rate with achievable peak plasma levels after 1 h 12 min following treatment and after 1 h in the brain[67]. FAAH inhibition in the brain was rapidly achieved in ≤ 1 h, and tolerated at > 90 % within 12 h and > 60 % between 12 and 24 h after an oral dose of 10 mg/kg of URB597.

The maximal plasma concentrations of URB597 attained within 15 min and subsequent long lasting exhibition of inhibition of FAAH may be accounted for by its irreversible covalent nature of binding and good bioavailability. The favourable pharmacological properties of URB597 have continually been reported[15,66]. These underscore the value of FAAH as a target for innovative analgesic drug, moreso, probably, in combination with NSAIDs. Nevertheless, it is believed that the therapeutic potential of the entire endocannabinoid system, and more specifically FAAH, has not been fully explored. The number of disorders that may be treated via FAAH and regulation of the entire endocannabinoid system will likely continue to grow.

**Conclusion**

The OPA based fluorimetric method is suitable for high throughput screening for potency of various compounds and quick study of enzyme kinetics. URB597 exhibited higher potency at inhibiting FAAH hydrolysis of ODA than for ArDA and SyDA. In the same way, URB597 inhibited FAAH-2 metabolism of PFAMs in a rank order of ODA > ArDA ≈ SyDA, however, URB597 was more potent for FAAH-2 than FAAH. The $K_m$ and $V_{max}$ values obtained for ODA hydrolysis indicate that FAAH has higher affinity for ArDA than ODA. Our data suggest that URB597 inhibited FAAH hydrolysis of both ArDA and ODA in an uncompetitive manner.

**List of abbreviations**

AEA – Anandamide / *N*-Arachidonoyl ethanolamide
ArDA – Arachidonamide
$CB_1$ – Cannabinoid receptor 1
$CB_2$ – Cannabinoid receptor 2
COX-2 – Cyclooxygenase-2
FAAH (-1) – Fatty acid amide hydrolase (-1)
FAAH-2 – Fatty acid amide hydrolase-2
LOX – Lipoxygenase
MAGL – Monoacyl-glycerol lipase
NAAA – *N*-Acylethanolamine-hydrolysing acid amidase
NAE(s) – *N*-acyl ethanolamide(s)
ODA – Oleamide
OPA - O-Phthalaldehyde
P. buffer – Potassium Phosphate buffer
PFAM(s) – Primary fatty acid amide(s)
THC – $\Delta^9$-Tetrahydrocannabinol
URB597 – [3-(3-carbamoylphenyl) phenyl] N-cyclohexyl carbamate
PEI – Polyethylenimine
SEM – Standard Error of the Mean
TRPV1 – Transient receptor potential vanilloid type-1

**Conflict of Interest Notification**

The authors declare that they have no conflict of interests.

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