Design, Synthesis, and Characterization of α-Ketoheterocycles That Additionally Target the Cytosolic Port Cys269 of Fatty Acid Amide Hydrolase

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ABSTRACT: A series of α-ketooxazoles incorporating electrophiles at the C5 position of the pyridyl ring of 2 (OL-135) and related compounds were prepared and examined as inhibitors of fatty acid amide hydrolase (FAAH) that additionally target the cytosolic port Cys269. From this series, a subset of the candidate inhibitors exhibited time-dependent FAAH inhibition and noncompetitive irreversible inactivation of the enzyme, consistent with the targeted Cys269 covalent alkylation or addition, and maintained or enhanced the intrinsic selectivity for FAAH versus other serine hydrolases. A preliminary in vivo assessment demonstrates that these inhibitors raise endogenous brain levels of anandamide and other FAAH substrates upon intraperitoneal (i.p.) administration to mice, with peak levels achieved within 1.5−3 h, and that the elevations of the signaling lipids were maintained >6 h, indicating that the inhibitors effectively reach and remain active in the brain, inhibiting FAAH for a sustained period.

INTRODUCTION

Because of the therapeutic potential of inhibiting fatty acid amide hydrolase (FAAH)1,2 for the treatment of pain,3−4 inflammatory,5 or sleep disorders,6 there is a continuing interest in the development of selective inhibitors of the enzyme.7 The distribution of FAAH is consistent with its role in regulating signaling fatty acid amides8−10 including anandamide (1a)11 and oleamide (1b)12,13 at their sites of action (Figure 1). Although FAAH is a member of the amidase signature family of serine hydrolases for which there are a number of prokaryotic enzymes, it is the only well-characterized mammalian enzyme bearing the family’s unusual Ser−Ser−Lys catalytic triad.14,15

Figure 1. Substrates of fatty acid amide hydrolase.

Early studies following the initial identification of the enzyme led to the disclosure of a series of substrate-inspired inhibitors that were used to characterize the enzyme as a serine hydrolase.16−22 Subsequent studies disclosed several classes of inhibitors that provide opportunities for the development of inhibitors with therapeutic potential. These include the reactive aryl carbamates and ureas23−31 that irreversibly carbamylate the FAAH active site catalytic serine.32 A second, and one of the earliest classes, is the α-ketoheterocycle-based inhibitors33−44 that bind to FAAH by reversible hemiketal formation with the active site catalytic serine. Many of these reversible, competitive inhibitors have been shown to be selective for FAAH versus other mammalian serine hydrolases as well as efficacious analgesics in vivo.44,45 In these studies, 2 (OL-135)36 emerged as a potent (Kᵢ = 4.7 nM)36 and selective (>60−300 fold)19 prototypical FAAH inhibitor that induces analgesia and increases endogenous anandamide levels.45 It lacks significant off-site target activity, does not bind cannabinoid (CB1 or CB2) or vanilloid (TRP) receptors, and does not significantly inhibit common P450 metabolism enzymes or the human ether-a-go-go related gene product (hERG). The analgesic effects of 2 are observed without the respiratory depression or chronic dosing desensitization characteristic of opioid administration or the increased feeding and decreased motor control characteristic of cannabinoid (CB) agonist administration.45 It possesses a relatively short duration of in vivo activity relative to irreversible inhibitors, although further conformational constraints in the C2 acyl chain of 2 have provided inhibitors that are not only orally active but also exhibit extended durations of in vivo activity.44
Complementary to a series of systematic structure—activity relationship (SAR) studies on 2 exploring substitution of the central oxazole, the C2 acyl side chain, and the central heterocycle,33−46 the X-ray characterization of inhibitor-bound complexes defined key features that impact inhibitor affinity and selectivity.47−50 These include not only the Ser241 hemiketal formation with the inhibitor electrophilic carbonyl and its interaction with the enzyme oxyanion hole but also an unusual Ser217-mediated OH−π H-bond to the activating heterocycle and the key anchoring interaction of the terminal phenyl group of the C2 acyl chain. The structural studies also revealed that Cys269 is located adjacent to C5 of the inhibitor pyridine substituent, which in turn is engaged in a series of intricate interactions in the enzyme cytosolic port.51,52 Herein, we report results of a systematic study of candidate inhibitors containing modifications at the pyridyl C5-position of 2 and related inhibitors that in principle could covalently trap this proximal Cys269 to provide inhibitors that alkylate or cross-link the FAAH active site. In turn, this could be expected to enhance their potency, potentially enhance their selectivity, and extend their in vivo duration of action (Figure 2). Herein, we detail the systematic inhibitor modifications that led to the discovery and characterization of such inhibitors52 and the unexpected trends that the additional strategically placed electrophiles display.

RESULTS AND DISCUSSION

Chemistry. The series 1 analogues (3−22) were accessed from 5-((tributylstannyl)oxazole 1f36 by Stille coupling53 with the appropriate 2-chloro- or 2-bromopyridine (Scheme 1). This was followed typically by TBS ether deprotection (Bu4NF) and oxidation of the liberated alcohol with Dess−Martin periodinane (DMP)54 to provide the corresponding α-ketoheterocycles: 3, 7, 9, 14, and 18−22. The remaining inhibitors were accessed by further modification of the pyridyl C5 substituent (Scheme 1).

The second series, in which the pyridine of 2 is replaced with an alkyl linker to the pendant electrophile, was accessed by Sonogashira coupling of 5-bromooxazole 1f43 with the appropriate alkyne (Scheme 2). The alkyne intermediate was reduced to the corresponding alkane with H2 and palladium on carbon or palladium hydroxide. This was followed by TBS ether deprotection (Bu4NF) and oxidation of the liberated alcohol with Dess−Martin periodinane (DMP) to yield the series 2 C5-substituted oxazoles: 23 and 28. Further elaboration of the terminal electrophile (R group) yielded the remaining compounds: 24−27 and 29−32.

Enzyme Inhibition. The initial characterization of the candidate inhibitors and their comparison with 2 was conducted using purified recombinant rat FAAH (rFAAH) expressed in Escherichia coli55 at 20−23 °C as previously disclosed.38 The initial rates of hydrolysis (>10−20% reaction) were monitored using enzyme concentrations below the initially measured K_i values by following the breakdown of 14C-oleamide, and K_i values were established as previously described (Dixon plot).

Series 1 was developed directly on the basis of 2 (K_i = 4.7 nM), placing a potential thiol-capturing electrophile at the S-position of the pyridine ring (5−8, 11, 12, 14, and 16−22). Thioesters 5 and 16 were expected to be the most straightforward traps for the Cys269 thiol by thioester exchange. Without preincubation of the inhibitors with the enzyme, these inhibitors along with their precursors (3−22) were tested for binding and inhibition of rFAAH (Figure 3). All display potencies similar to 2, exhibiting K_i values in the low nanomolar range. In series 2, the pyridine ring was replaced by an alkyl chain of appropriate length capped with the thiol-engaging moiety. As modeled, this flexible linker is able to reach through the cytosolic pocket and place the potentially reactive electrophile proximal to Cys269. Like the series 1 inhibitors and without preincubation with the enzyme, all series 2 inhibitors exhibited effective FAAH inhibition with potencies that approach or match that of 2 (Figure 3).

Time-Dependent Enzyme Inhibition. Because the Cys269 alkylation was expected to be slow relative to the rapid hemiketal formation, the time-dependent inhibition of FAAH was examined. This was accomplished by preincubation of the inhibitors with recombinant rFAAH for a period of 1−6 h. As previously observed, reversible, competitive inhibitor 2 does not display time-dependent inhibition of FAAH, and its K_i value remains unchanged with the enzyme–inhibitor preincubation times of 0−6 h (Figure 4). In contrast, a select subset of inhibitors (11, 14, 17, and 20−22) in series 1 exhibited significant increases in potency, displaying 2−20-fold improvements in K_i over the same time period, consistent with slow irreversible inhibition of FAAH. Surprisingly, thioesters 5 or 16 did not exhibit this time-dependent increase in enzyme inhibition potency. Similarly, chloride 12 was found to be relatively nonpotent and insensitive to preincubation with the enzyme, whereas the corresponding

Figure 2. Inhibitor series examined.
bromide 11 was initially more potent and exhibited the most pronounced time-dependent increase in potency of all inhibitors. Both nitrile 7 and its imidate 8, where the candidate electrophile is attached directly to the pyridyl ring, did not display time-dependent increases in potency, whereas both the homologated nitrile 14 and its imidate 17, where a methylene spacer separates the electrophile and pyridyl ring, did exhibit increases in potency with the enzyme–inhibitor preincubation. Of the series of inhibitors that might be expected to serve as Michael acceptors for a thiol conjugate addition (18 – 22), including the α,β-unsaturated ester 18 and nitrile 19, only those bearing the weaker activating substituents (20 – 22 vs 18 and 19) that would be expected to react slower and to be intrinsically less reversible displayed the exceptionally potent and time-dependent FAAH inhibition improvements. Notably and throughout this series, it was not the anticipated electrophiles that exhibited the time-dependent inhibition of FAAH characteristic of a slow irreversible inhibitor, but rather it was a less-well-recognized alternative (14 and 17 vs 16, 11 vs 12, and 20 – 22 vs 18 – 19). Finally, no inhibitor in series 2 that bears the flexible linker to the second electrophile displayed the time-dependent increases in potency, indicating that the conformationally restricted place-
ment of the second electrophile is important to observation of the targeted alkylation. For the inhibitors that displayed time-dependent increases in inhibitor potency, enzyme activity did not recover after this time period and is indicative of irreversible enzyme inhibition.

Lineweaver–Burk Kinetic Analysis. The compounds that demonstrated time-dependent improvements in potency were further investigated by Lineweaver–Burk kinetic analysis. In previous studies, α-ketoheterocycle inhibitors including 2 were shown to display well-behaved competitive, reversible inhibition kinetics. Despite expectations but consistent with the lack of time-dependent FAAH inhibition, Lineweaver–Burk kinetic analysis of thioesters 5, 16, and 25 after 3 h preincubation with the enzyme confirmed that they also behave as reversible, competitive inhibitors, analogous to 2 and related α-ketoheterocycle inhibitors (Figure 5). Thus, despite the expectations of a facile transthioesterification with Cys269, the thioesters exhibit enzyme inhibition characteristic of reversible inhibitors, suggesting that reaction with Cys269 does not occur.

**Figure 3.** Enzyme inhibition.

| R = compd | $K_i$ (nM) | R = compd | $K_i$ (nM) |
|-----------|------------|-----------|------------|
| MeO       | 3 3.1      |              |
| O       | 4 8.1      | 14 1.5      |
| O        | 5 2.0      | 15 4.0      |
| O        | 6 2.2      | 16 3.6      |
| O        | 7 9.9      | 17 5.1      |
| O        | 8 6.1      | 18 11.1     |
| MOMOMO    | 9 10.1     | 19 2.1      |
| HO        | 10 3.2     | 20 2.1      |
| Br        | 11 3.1     | 21 2.0      |
| Cl        | 12 22.4    | 22 3.0      |
| MeS       | 13 11.1    | 60 0.15     |

| R = compd | $K_i$ (nM) | R = compd | $K_i$ (nM) |
|-----------|------------|-----------|------------|
| MeO       | 23 1.8     |              |
| O        | 24 23.8    | 28 24.1     |
| O        | 25 1.8     | 29 10.1     |
| O        | 26 14.6    | 30 6.1      |
| NC        | 27 4.8     | 31 5.2      |

| R = compd | $K_i$ (nM) | R = compd | $K_i$ (nM) |
|-----------|------------|-----------|------------|
| MeS       | 25 1.8     | 26 1.4     |
| NC        | 27 4.8     | 27 4.4     |
| Br        | 30 6.1     | 30 5.9     |
| Cl        | 32 5.2     | 32 6.4     |

**Figure 4.** Time-dependent inhibition. $K_i$ values were measured after 0–6 h inhibitor preincubation with rFAAH.

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Significantly, thioesters 5 and 25 were recovered unchanged from the assay buffer (6 h) and from enzymatic assays (5), indicating that they are not undergoing chemical hydrolysis or transient enzyme adduct formation and subsequent hydrolysis under the conditions of the assay.

In contrast, the inhibitors that demonstrated a time-dependent increase in inhibitor potency also exhibited noncompetitive inhibition of FAAH when preincubated with the enzyme for 3 h prior to Lineweaver–Burk kinetic analysis (Figure 6). This is expected of irreversible enzyme inhibition and consistent with Cys269 alkylation or addition to the pendant electrophile. In the case of 11, this entails Cys269 thiol nucleophilic displacement of the benzylic bromide to provide the corresponding thioether, and its structure has been confirmed by X-ray analysis of the inhibitor bound to FAAH.52 The noncompetitive enzyme inhibition presumably entails thiol nucleophilic addition to the electrophile to provide the Cys269-linked thioimidate for 14 and 17, and it presumably involves an apparent irreversible thiol conjugate addition to 20–22. Interestingly, both the αβ-unsaturated nitrile 19 and ester 18, which do not exhibit time-dependent increases in inhibitor activity or the potent $K_i$ values consistent with irreversible inhibition, displayed mixed kinetics, exhibiting competitive inhibition at low inhibitor concentrations and noncompetitive inhibition at high concentrations. Presumably, this indicates that the thiol conjugate addition products derived from 18 and 19 are either formed less effectively or, more likely, that they may be sufficiently reversible at 23 °C to less effectively trap Cys269 as an apparent irreversible inhibitor of the enzyme.

**Irreversible Enzyme Inhibition.** Dialysis dilution (4 °C, 18 h, 370-fold) of the FAAH-inhibitor mixture following 3 h of preincubation with 2 restored full enzyme activity, consistent with its reversible enzyme inhibition, whereas the mixtures containing 11, 14, and 17–22 remained relatively unchanged, failing to restore FAAH activity, indicative of irreversible enzyme inhibition under the conditions monitored (4 °C, pH 9, Figure 7). It is notable that 14 and 17 (not shown), which presumably form a Cys269 thioimidate adduct, do not appear to be even slowly reversible under these conditions. Similarly, 20–22 displayed irreversible inhibition of FAAH, consistent with their time-dependent, noncompetitive enzyme inhibition. Interestingly, dialysis dilution at 4 °C also did not restore enzyme activity with both the αβ-unsaturated nitrile 19 and ester 18, which do not exhibit time-dependent FAAH inhibition and displayed concentration-dependent mixed competitive/noncompetitive

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**Figure 5.** Lineweaver–Burk kinetic analysis of 5, 16, and 25 demonstrate reversible, competitive inhibition.

**Figure 6.** Lineweaver–Burk analysis demonstrates noncompetitive FAAH inhibition for (A) 11, (B) 14, (C) 17, (D) 20, (E) 21, and (F) 22.
kinetics in the Lineweaver–Burk analysis at 23 °C. This suggests that their inhibition of FAAH following the 3 h incubation (22 °C) is not reversible at 0 °C. Unfortunately, the reversibility of 18 and 19 at 23 °C could not be established because of the instability of FAAH at 23 °C over the dialysis time frame.

Inhibitor Selectivity. The selectivity of the time-dependent, irreversible FAAH inhibitors 17 and 20–22 were examined along with 11 and 14 that were recently disclosed57 using activity-based protein profiling (ABPP) of the serine hydrolases.57 ABPP methods permit the testing of serine hydrolases in their native state and eliminate the need for their recombinant expression, purification, and the development of specific substrate assays. Because inhibitors are screened against many enzymes in the proteome in parallel, both relative potency and selectivity can be simultaneously evaluated. Previous studies19,37,52 have shown that the α-ketoheterocycle class of inhibitors are selective for FAAH, although four enzymes have emerged as potential competitive targets: triacylglycerol hydrolase (TGH), α/β hydrolase containing domain 6 (ABHD6), monoacylglycerol lipase (MAGL), and the membrane-associated hydrolase KIAA1363. Each inhibitor was tested for its effects on the fluorophosphonate (FP)-rhodamine probe labeling of serine hydrolases in the mouse brain (contains KIAA1363, MAGL, and ABHD6) and heart membrane (contains TGH) proteome at concentrations ranging from 10 nM to 100 μM. The selectivity assessments were conducted following 6 h inhibitor incubation with the proteomes and all inhibitors showed superb selectivity for FAAH over KIAA1363 and ABHD6 (>104-fold), excellent selectivity over MAGL (>200-fold), and good selectivity over TGH (Figure 8).

Preliminary in Vivo Characterization. In initial efforts to screen for in vivo inhibition of FAAH and its subsequent pharmacological effects, the set of inhibitors displaying the time-dependent, irreversible FAAH inhibition (11, 14, 17, and 20–22) were examined alongside of 2 for their ability to increase the endogenous levels of a series of lipid amide signaling molecules that are substrates for FAAH in both the brain (CNS effect) and liver (peripheral effect, not shown). Thus, the effects of the inhibitors on the endogenous levels of the FAAH substrates anandamide (AEA), oleoyl ethanolamide (OEA), and palmitoyl ethanolamide (PEA) were measured. Notably, it is the increase in endogenous levels of anandamide and its subsequent action at cannabinoid (CB1 and CB2) receptors that are thought to be responsible for the analgesic and anti-inflammatory effects of FAAH inhibitors. The effects were established 3 h following intraperitoneal (i.p.) administration of inhibitor in three mice per time point for an initial screen (30 mg/kg). Significantly, increases in endogenous levels of anandamide in the brain requires >90% inhibition of FAAH for in vivo enzyme inhibition.58 With the exception of imidate 17, which matched the increased anandamide levels observed with 2 after 3 h, each of the additional inhibitors proved to be roughly equivalent (11, 14, and 20 > 21 and 22), increasing anandamide levels approximately 2-fold over that of 2 and approximately 3-fold over vehicle treatment (Figure 9).

With PEA and OEA, which show significant enhancements in endogenous levels with partial enzyme inhibition and are less sensitive to the extent of FAAH inhibition, all of the inhibitors that displayed time-dependent, irreversible FAAH inhibition matched or exceeded the activity of 2, producing elevations of 3–12-fold over vehicle. Of these, both bromide 11 and nitrile 14 exhibited the largest increases. As a result, more detailed dose- and time-dependent studies of 11 and 14 were conducted as reported elsewhere.52 The results of these studies revealed that they cause accumulation of all three lipid amides in the brain with peak levels achieved within 1.5–3 h, that these elevations exceed those achieved with the reversible inhibitor 2, that these elevations are maintained >6 h (vs 2–3 h for 2), consistent with irreversible enzyme inhibition, and that they exhibit long acting in vivo activity in a mouse model of neuropathic pain.52

**CONCLUSIONS**

The design, synthesis, and characterization of α-ketoheterocycles that additionally target the remote Cys269 nucleophile found in the cytosolic port of FAAH59 provided inhibitors that slowly react with the enzyme nucleophile, effectively providing time-dependent, irreversible inhibitors of the enzyme that maintain or enhance their selectivity for FAAH over other serine hydrolases. The electrophiles capable of targeting Cys269 were incorporated as a C5 substituent on the pyridyl group of the 5-(pyrid-2-yl) oxazole of 2 and ranged from the reactive benzylic bromide 11 to the otherwise benign nitrile 14. The irreversible inhibitors of FAAH displayed an expected sensitivity to the position of the electrophile introduction, but those that were successful exhibited surprising trends in apparent reactivity toward Cys269 that would not be easily predicted. A preliminary in vivo characterization of the identified irreversible FAAH inhibitors could not be established because of the instability of...
inhibitors confirmed their ability to raise endogenous brain levels of the enzyme substrates, including anandamide, in mice to a greater extent (>2-fold) and for a longer duration (>6 h) than the reversible α-ketoheterocycles on which they are based. Two of these (11 and 14) were characterized in greater detail, as reported elsewhere, along with their long acting in vivo efficacy in a mouse model of neuropathic pain.52

**EXPERIMENTAL SECTION**

**FAAH Inhibition.** 14C-labeled oleamide was prepared from 14C-labeled oleic acid as described.13 The truncated rat FAAH (rFAAH) was expressed in *E. coli* and purified as described,52 and the purified recombinant rFAAH was used in the inhibition and reversibility assays unless otherwise indicated. The purity of each tested compound (>95%) was determined on an Agilent 1100 LC/MS instrument using a ZORBAX SB-C18 column (3.5 mm, 4.6 mm × 50 mm, with a flow rate of 0.75 mL/min and detection at 220 and 253 nm) with a 10–98% acetonitrile/water/0.1% formic acid gradient (two different gradients). The inhibition assays were performed as described.56 The enzyme reaction was initiated by mixing 1 nM rFAAH (800, 500, or 200 pM rFAAH for inhibitors with K_i ≤ 1–2 nM) with 20 μM 14C-labeled oleamide in 500 μL of reaction buffer (125 mM TrisCl, 1 mM EDTA, 0.2% glycerol, 0.02% Triton X-100, and 0.4 mM Hepes, pH 9.0) at room temperature in the presence of three different concentrations of inhibitor. The enzyme reaction was terminated by transferring 20 μL of the reaction mixture to 500 μL of 0.1 N HCl at three different time points. The 14C-labeled oleamide (substrate) and oleic acid (product) were extracted with EtOAc and analyzed by TLC as detailed.13 The K_i of the inhibitor was calculated using a Dixon plot. Lineweaver–Burk kinetic analysis was performed as described,56 confirming competitive, reversible inhibition for 5, 16, and 25 and noncompetitive inhibition for 11, 14, 17, and 20–22 (Figures 5 and 6).

**Reversibility of FAAH Inhibition (Dialysis).** The reversibility of FAAH inhibition by 2, 11, 14, and 17–22 was assessed by dialysis dilution using purified recombinant rFAAH. The enzyme was placed in 15 mL of FAAH assay buffer (125 mM Tris, 1 mM EDTA, 0.2% glycerol, 0.02% Triton X-100, and 0.4 mM Hepes, pH 9.0). A 3 μL aliquot of membrane homogenate was used for each sample dialyzed. The dialysis experiment was performed in the pre dialysis mix or at near the apparent IC_{50}. The final assay inhibitor concentrations used were 100 nM, 2, 18, and 19; 80 nM, 11, 14, 21, and 22; and 150 nM, 20. Samples were preincubated with the enzyme for 3 h at room temperature (22 °C) before 300 μL was removed and assayed in triplicate in a FAAH activity assay. The remaining sample (2.7 mL) was injected into a dialysis cassette employing a 10 000 MW cutoff membrane. The mixture was dialyzed against 1 L of PBS at 4 °C on a stir plate for 18 h. The postdialysis FAAH activity was assessed by assaying 300 μL samples taken from the dialysis cassettes in triplicate. FAAH activity is expressed as a percentage of vehicle-treated FAAH (DMSO alone) and is shown in Figure 7.

**Competitive ABPP of FAAH Inhibitors with FP-Rhodamine.** Mouse tissues were Dounce-homogenized in PBS buffer (pH 8.0), and membrane proteomes were isolated by centrifugation at 4 °C (100 000 g, 45 min), washed, resuspended in PBS buffer, and adjusted to a protein concentration of 1 mg/mL. Proteomes were preincubated with inhibitors (10–100 000 nM, DMSO stocks) for 6 h and then treated with FP-rhodamine (100 nM, DMSO stock) at room temperature for 10 min. Reactions were quenched with SDS-PAGE loading buffer, subjected to SDS-PAGE, and visualized in-gel using a flatbed fluorescence scanner (MiraBio). Labeled proteins were quantified by measuring integrated band intensities (normalized for volume); control samples (DMSO alone) were considered to have 100% activity. IC_{50} values (n = 2–4) were determined from dose–response curves using Prism software and are reported in Figure 8.

**In Vivo Pharmacodynamic Studies with Inhibitors.** Inhibitors were prepared as a saline–emulphor emulsion for intraperitoneal (i.p.) administration by vortexing, sonicating, and gently heating neat compound directly in an 18:1:1 v/v/v solution of saline/ethanol/emulphor. Male C57Bl/6J mice (<6 months old, 20–28 g) were administered inhibitors in saline–emulphor emulsion or an 18:1:1 v/v/v saline/emulphor/ethanol vehicle i.p. at a volume of 10 μL/g weight. After the indicated amount of time (1, 3, or 6 h), mice (n = 3 for each compound at each time point) were anesthetized with isoflurane and killed by decapitation. Total brains (∼400 mg) and a portion of the liver (∼100 mg) were removed and flash frozen in liquid N_2. Animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of The Scripps Research Institute.

**Measurement of Brain Lipids.** Tissue was weighed and subsequently Dounce-homogenized in 2:1:1 v/v/v CHCl_3/MeOH/Tris pH 8.0 (8 mL) containing standards for lipids (50 pmol of d_6-PEA, 2 pmol of d_5-AEA, and 10 nmol of pentadecanoic acid). The mixture was...
vortexed and then centrifuged (1400g, 10 min). The organic layer was removed, dried under a stream of N₂, and resolubilized in 2:1 v/v CHCl₃/MeOH (120 µL), and 10 µL of this resolubilized lipid was injected onto an Agilent G6410B QQQ instrument. LC separation was achieved with a Gemini reverse-phase C18 column (50 mm, Phenomenex) together with a precolumn (C18, 3.5 µm, 2 mm × 20 mm). Mobile phase A was composed of 95:5 v/v H₂O/MeOH, and mobile phase B was composed of 65:35:5 v/v/v p-POH/MeOH/H₂O. The flow rate for each run started at 0.1 mL/min with 0% B. At 5 min, the solvent was immediately changed to 60% B with a flow rate of 0.4 mL/min and increased linearly to 100% B over 10 min. This was followed by an isocratic gradient of 100% B for 5 min at 0.5 mL/min before equilibrating for 3 min at 0% B at 0.5 mL/min (23 min total per sample). MS analysis was performed with an electrospray ionization (ESI) source. The following MS parameters were used to measure the indicated metabolites in positive mode (precursor ion, product ion, collision energy in V): AEA (348, 62, 11), OEA (326, 62, 11), PEA (300, 62, 11), d₅-AEA (352, 66, 11), and d₅-PEA (304, 62, 11). The capillary was set to 4 kV, the ionization source was set to 100 V, and the delta EMV was set to 0. Lipids were quantified by measuring under the peak in comparison to the standards (n = 3 for each inhibitor at each time point).

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**ABBREVIATIONS USED**

AA, arachidonic acid; ABHD6, αβ-γ-diacetil hydrolyase containing domain 6; ABPP, activity-based protein profiling; AEA, anandamide; CB, cannabinoid; DPP, D-α-tocopherol; FAAH, fatty acid amide hydrolase; i.p., intraperitoneal; MAGL, monoacylglycerol lipase; OEA, oleoyl ethanolamide; PEA, palmitoyl ethanolamide; TBS, tert-butyldimethylsilyl; TGH, triacylglycerol hydrolase; TBS, tert-butyldimethylsilyl; TGH, triacylglycerol hydrolase; TGF, triacylglycerol hydrolase.

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**ASSOCIATED CONTENT**

Supporting Information

Full experimental details and characterization of the candidate inhibitors, inhibitor purities, and enzyme inhibition measurement standard deviations for Figures 3, 4, and 8. This material is available free of charge via the Internet at http://pubs.acs.org.

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**ABBREVIATIONS USED**

AA, arachidonic acid; ABHD6, αβ-γ-diacetil hydrolyase containing domain 6; ABPP, activity-based protein profiling; AEA, anandamide; CB, cannabinoid; DPP, D-α-tocopherol; FAAH, fatty acid amide hydrolase; i.p., intraperitoneal; MAGL, monoacylglycerol lipase; OEA, oleoyl ethanolamide; PEA, palmitoyl ethanolamide; TBS, tert-butyldimethylsilyl; TGH, triacylglycerol hydrolase.

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**ASSOCIATED CONTENT**

Supporting Information

Full experimental details and characterization of the candidate inhibitors, inhibitor purities, and enzyme inhibition measurement standard deviations for Figures 3, 4, and 8. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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