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Published on Web 07/06/2006

The Putative Endocannabinoid Transport Blocker LY2183240 Is a Potent Inhibitor of FAAH and Several Other Brain Serine Hydrolases

Jessica P. Alexander and Benjamin F. Cravatt*

Contribution from the Skaggs Institute for Chemical Biology and Departments of Cell Biology and Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

Received April 29, 2006; E-mail: cravatt@scripps.edu

Abstract: How lipid transmitters move within and between cells to communicate signals remains an important and largely unanswered question. Integral membrane transporters, soluble lipid-binding proteins, and metabolic enzymes have all been proposed to collaboratively regulate lipid signaling dynamics in vivo. Assignment of the relative contributions made by each of these classes of proteins requires selective pharmacological agents to perturb their individual functions. Recently, LY2183240, a heterocyclic urea inhibitor of the putative endocannabinoid (EC) transporter, was shown to disrupt the cellular uptake of the lipid EC anandamide and promote analgesia in vivo. Here, we show that LY2183240 is a potent, covalent inhibitor of the EC-degrading enzyme fatty acid amide hydrolase (FAAH). LY2183240 inactivates FAAH by carbamylation of the enzyme's serine nucleophile. More global screens using activity-based proteomic probes identified several additional serine hydrolases that are also inhibited by LY2183240. These results indicate that the blockade of anandamide uptake observed with LY2183240 may be due primarily to the inactivation of FAAH, providing further evidence that this enzyme serves as a metabolic driving force that promotes the diffusion of anandamide into cells. More generally, the proteome-wide target promiscuity of LY2183240 designates the heterocyclic urea as a chemotype with potentially excessive protein reactivity for drug design.

Introduction

The fatty acid amide class of lipid transmitters modulates a wide range of physiological and pathological processes.[1,2] Prominent bioactive fatty acid amides include the endogenous cannabinoid *N*-arachidonoyl ethanolamine (anandamide),[3] the sleep-inducing substance 9*Z*-octadecenamide (oleamide),[4] and the anti-inflammatory substance *N*-palmitoylethanolamine (PEA).[5] In contrast to classical neurotransmitters, which are stored in vesicles prior to release, lipid messengers such as the fatty acid amides are thought to be enzymatically produced "on-demand" (i.e., at the moment of their intended action).[6] Termination of fatty acid amide signaling also requires the action of enzymes to degrade these molecules. Prominent among these catabolic enzymes is the integral membrane protein fatty acid amide hydrolase (FAAH).[7,8] Several lines of evidence support a key role for FAAH in the control of fatty acid amide function in vivo. For example, the inactivation of FAAH by genetic[9] or pharmacological[10] techniques leads to highly elevated levels of anandamide and related fatty acid amides in the nervous system and concomitant CB1-dependent analgesic and anxiolytic phenotypes.

The localization of FAAH to intracellular membrane compartments of neurons[11,12] has raised a provocative question: how are fatty acid amides such as anandamide delivered to the enzyme for degradation? One model invokes the action of a plasma membrane-associated transporter that promotes the uptake of extracellular anandamide by facilitated diffusion.[13] Evidence for the existence of this putative transporter includes the saturability and temperature dependence of anandamide uptake, as well as its disruption by pharmacological agents. However, each of these criteria has also been subject to alternative interpretations.[14,15] For example, anandamide binds

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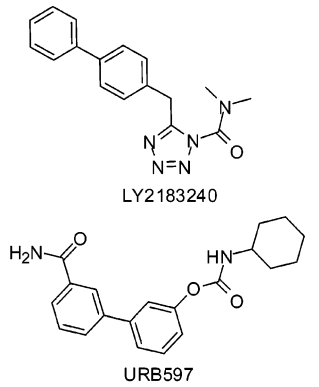

Figure 1. Structures of the putative anandamide transport blocker LY2183240 and the FAAH inhibitor URB597.

to culture dishes without cells, especially in the absence of serum albumin, and this process is saturable, temperature-dependent, and sensitive to chemical inhibitors.[16,17] Likewise, most of the transport inhibitors described to date also block FAAH,[18−20] suggesting that this enzyme may create a metabolic driving force for the cellular uptake of anandamide by simple diffusion.[18,21] Consistent with this model, cells that express high levels of FAAH show greatly accelerated rates of anandamide uptake.[22−24] Other studies have indicated that the cellular uptake of free anandamide can appear saturable due to the presence of an unstirred water layer adjacent to the cell membrane that impedes the uptake of this lipid.[21,25]

The molecular characterization of a putative anandamide transporter would be facilitated by the creation of potent and selective agents that perturb its function. Toward this end, a novel blocker of anandamide uptake, LY2183240 (Figure 1), was recently described that displays unprecedented potency (IC_{50} value of 270 pM in a cellular assay).[24] LY2183240 also showed impressive pharmacological activity in vivo, raising anandamide levels and reducing pain responses in the formalin test.[24] Unlike most transport inhibitors, LY2183240 is not a structural analogue of anandamide, suggesting that it might avoid interactions with other natural protein partners of this lipid (e.g., cannabinoid receptors, FAAH). On the other hand, LY2183240 bears some structural resemblance to certain FAAH inhibitors, such as URB597 (Figure 1), including a biraryl substituent and a potentially reactive carbonyl. Here, we show that LY2183240 is, in fact, an extremely potent inhibitor of FAAH both in vitro and in vivo. LY2183240 inactivates FAAH by covalently labeling the enzyme's serine nucleophile. Functional proteomic screens identified several additional serine hydrolases that were also inactivated by LY2183240, indicating that the compound possesses rather promiscuous activity against this large and diverse enzyme class.

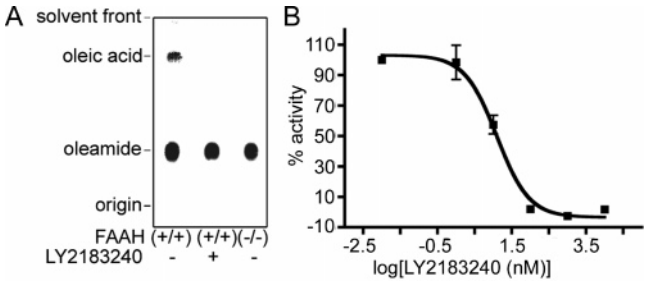

Figure 2. LY2183240 is a potent inhibitor of FAAH. (A) TLC plate showing conversion of ^{14}C-oleamide to ^{14}C-oleic acid by FAAH(+/+) brain membranes and its blockade by LY2183240 (1 μM). Control reactions with brain tissue from FAAH (−/−) mice confirm that the observed activity is due to FAAH. (B) Concentration-dependent inhibition of FAAH by LY2183240. From this curve, an estimated IC_{50} value of 12.4 nM (8.3−18.6 nM, 95% confidence limits) was measured.

Results

LY2183240 Is a Potent, Covalent Inhibitor of FAAH and Several Other Brain Serine Hydrolases. In the original report describing LY2183240 as a blocker of anandamide transport, this compound was shown to bind to cells that lack FAAH, suggesting that it may target a distinct protein(s).[24] However, direct interactions between LY2183240 and FAAH were not investigated. To address this important question, we incubated brain membranes with LY2183240 for 10 min and then tested for residual FAAH activity using a ^{14}C-substrate assay. LY2183240 potently inhibited FAAH activity with an IC_{50} value of 12.4 nM (Figure 2). This value approaches the estimated concentration of FAAH protein present in the brain proteome, as judged by quantitative labeling with a fluorophosphonate-rhodamine (FP-rhodamine) active site-directed probe,[26,27] suggesting that LY2183240 may titrate the enzyme in these reactions.

Considering that LY2183240 possesses a potentially reactive heterocyclic urea group, we next tested if this agent might inactivate FAAH by a covalent mechanism. Purified, recombinant rat FAAH protein (~23 μM) was incubated with or without LY2183240 (35 μM, ~1.5 equiv) for 60 min, after which the reaction mixtures were subjected to tryptic digestion and MALDI-TOF mass spectrometry (MS) analysis. A new mass signal was observed for the LY2183240-treated sample that corresponded to the mass of the FAAH tryptic peptide amino acids (AA) 213−243, which contains the FAAH nucleophile S241,[28,29] modified by the C(O)*N*-dimethyl substituent of the inhibitor (Figure 3). These results indicate that LY2183240 covalently inhibits FAAH by carbamylation of the enzyme's serine nucleophile, analogous to the mechanism of action of URB597 and other carbamate FAAH inhibitors.[30] Consistent with an irreversible mechanism of inhibition, LY2183240-treated

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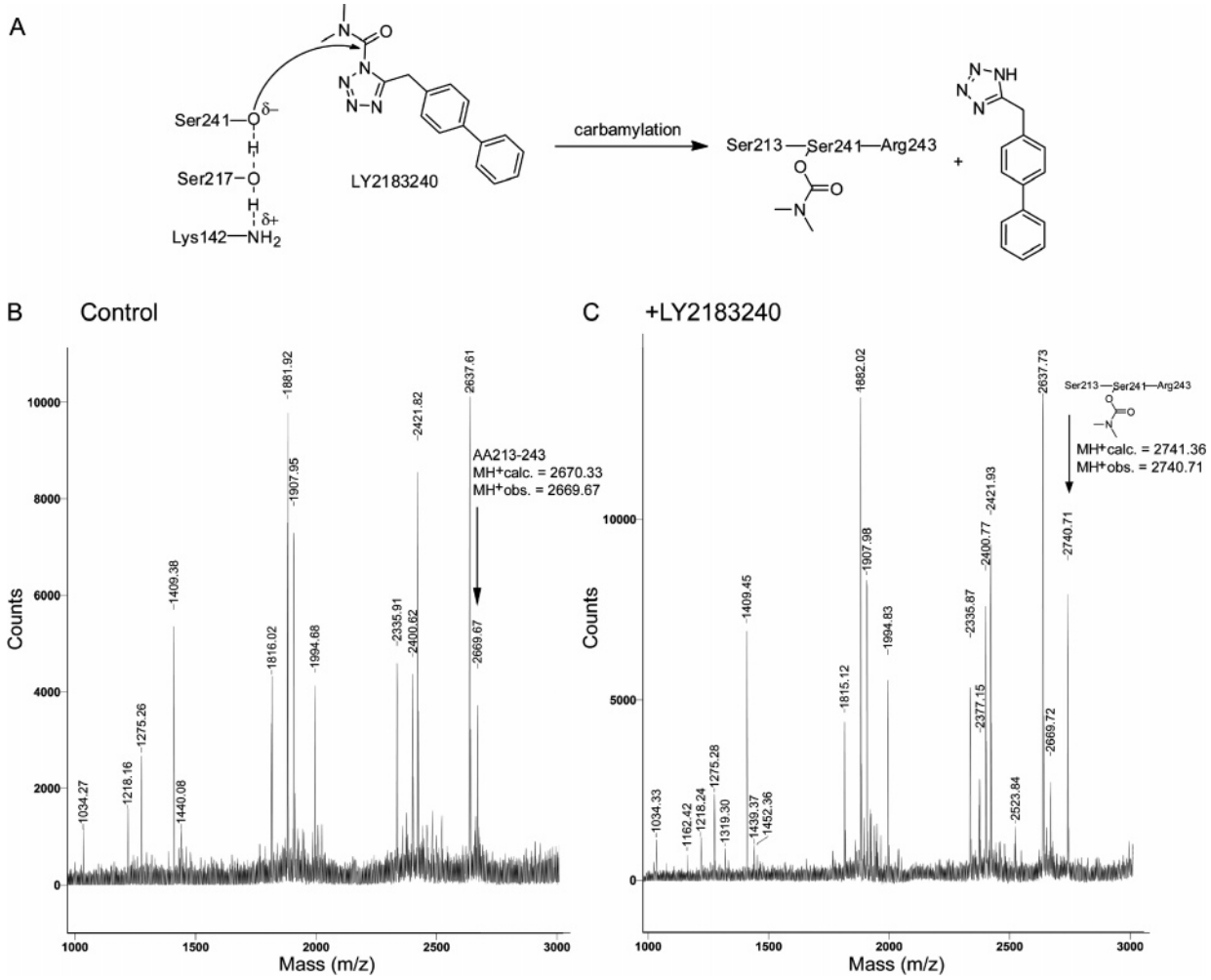

Figure 3. LY2183240 is a covalent inhibitor of FAAH. (A) Predicted mode of irreversible inactivation of FAAH by LY2183240, involving carbamylation of the enzyme's serine nucleophile (S241). (B) MALDI-MS mapping of a tryptic digest of purified recombinant FAAH, highlighting the tryptic peptide that contains S241 (AA213−243). (C) MALDI-MS mapping of a tryptic digest of FAAH pretreated with LY2183240, highlighting a new mass that corresponds to the AA213−243 peptide modified by one C(O)*N*-dimethyl molecule.

FAAH samples showed no evidence of recovery of activity for up to 24 h following gel filtration to remove free inhibitor (data not shown). Attempts to measure a k_2/K_i value for LY2183240 were complicated by the extremely rapid rate of inactivation (complete inactivation of FAAH with a 30-s preincubation), even at low inhibitor/FAAH ratios (10:1).

A search of the scientific and patent literature identified multiple reports of heterocyclic ureas as inhibitors of serine hydrolases, including peptidases[31] and hormone-sensitive lipase.[32] These previous studies led us to consider whether LY2183240 is a selective FAAH inhibitor or, alternatively, a more general inactivator of enzymes in the serine hydrolase superfamily. This question was addressed by treating brain proteomes with increasing concentrations of LY2183240 followed by the activity-based serine hydrolase probe FP-rhodamine.[26,27] In this competitive activity-based protein profiling (ABPP) screen, the selectivity of an inhibitor for members of the serine hydrolase class is determined by measuring reductions in FP-rhodamine labeling intensity for many enzymes in parallel.[33] Remarkably, LY2183240 blocked the FP-rhodamine labeling of several brain serine hydrolases with potencies comparable to that observed for FAAH (Figure 4A). These enzymes included the uncharacterized hydrolase KIAA1363,[33] as well as multiple unidentified enzymes with molecular masses between 25 and 35 kDa. We next pursued the characterization of enzymes targeted by LY2183240 in vivo.

LY2183240 Is an Inhibitor of FAAH and Several Other Serine Hydrolases in Vivo. LY2183240 has been reported at 10 mg/kg (intraperitoneal, ip) to produce analgesic effects in the formalin test of noxious pain, a phenotype that was correlated with a significant elevation in brain anandamide levels.[24] These molecular and behavioral effects are similar to those observed in FAAH-disrupted animals,[9,34] suggesting that pharmacologically active doses of LY2183240 may inhibit FAAH in vivo. We tested this premise by treating mice with LY2183240 (10 mg/kg, ip) for 90 min and then sacrificing these animals and characterizing their brain serine hydrolase activity profiles by labeling with FP-rhodamine. Parallel studies were conducted with URB597 (Figure 1, 10 mg/kg, ip), a covalent carbamate inhibitor of FAAH that shows good selectivity for this enzyme

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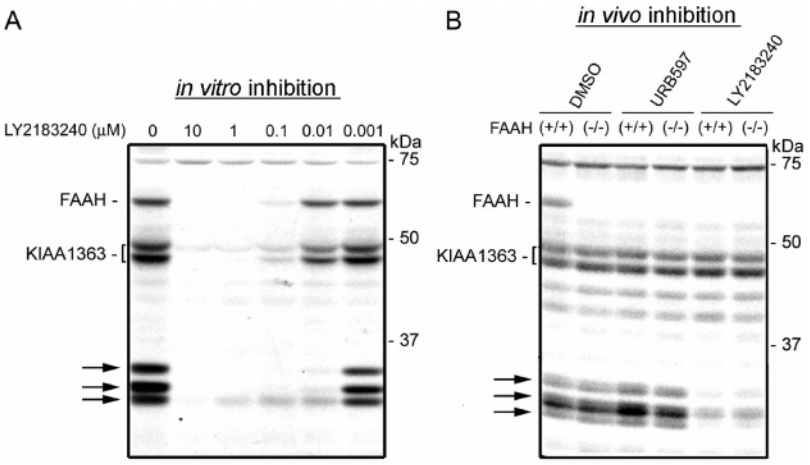

Figure 4. LY2183240 inhibits several brain serine hydrolases in vitro and in vivo. (A) Competitive ABPP profiles of brain membranes treated with LY2183240 (0.001−10 μM, 10 min) in vitro, followed by the serine hydrolase activity-based probe FP-rhodamine (0.1 μM, 10 min). The FP-labeling of several enzymes was blocked in a concentration-dependent manner by LY2183240, including FAAH, the uncharacterized hydrolase KIAA1363,[33] and multiple proteins between the molecular masses of 25−35 kDa (arrows). (B) Competitive ABPP profiles of brains isolated from mice treated with LY2183240 or URB597 (10 mg/kg, ip, 90 min). The FP-labeling of several enzymes was blocked by in vivo treatment with LY2183240, including FAAH, whose identity was confirmed by comparison with in vivo treatment of FAAH (−/−) mice, and a set of 25−35 kDa proteins (arrows). In contrast, in vivo treatment with URB597 selectively blocked the FP-labeling of FAAH.

in brain.[30] In vivo administration of LY2183240 was found to inactivate FAAH and multiple other serine hydrolases that reside in the molecular mass range of 25−35 kDa (Figure 4B, arrows). In contrast, URB597 selectively inactivated FAAH in brain tissue.

Identification and Characterization of Serine Hydrolase Targets of LY2183240. To identify the enzymes inactivated by LY2183240 in vivo, we analyzed tissues from vehicle- and inhibitor-treated mice using an advanced functional proteomic platform termed ABPP-multidimensional protein identification technology (ABPP-MudPIT).[35] In ABPP-MudPIT, proteomes are treated with a biotinylated activity-based probe (in this case, FP-biotin[36]), and probe-labeled enzymes are enriched by avidin chromatography and subjected to on-bead trypsin digestion and multidimensional LC−MS/MS analysis. Spectral counting methods are then used to quantify the relative levels of enzyme activities in different proteomic samples. ABPP-MudPIT analysis identified 40 serine hydrolases in mouse brain (Supporting Information Table 1). A number of these enzymes were partially or completely inhibited by LY2183240 in vivo, as judged by a reduction in spectral counts of greater than 2.5-fold relative to vehicle-treated controls (Table 1). These LY2183240-inhibited enzymes included FAAH (100% inhibited), α/β-hydrolase 6 (Abh6, >90% inhibited), and monoacylglycerol lipase (MAG lipase, >60% inhibited). In contrast, URB597 selectively inactivated FAAH in brain (Table 1).

Recombinant expression of Abh6 and MAG lipase in COS-7 cells confirmed that these enzymes were targets of LY2183240 (Figure 5), which inhibited their FP-rhodamine labeling with IC_{50} values of 0.09 and 5.3 nM, respectively (Table 2). Both of these enzymes migrated as 30−35 kDa proteins by SDS-PAGE, suggesting that they corresponded to the LY2183240 targets observed in this molecular mass range by gel-based ABPP (Figure 4). In contrast to these enzymes, which were inactivated by LY2183240 both in vitro and in vivo, KIAA1363 was only inhibited by LY2183240 in vitro (compare parts A and B of Figure 4). Why KIAA1363 shows resistance to LY2183240 in vivo remains unclear, especially considering that LY2183240

Table 1. Levels of Representative Serine Hydrolase Activities in Brains from Mice Treated with LY2183240 (10 mg/kg, ip), URB597 (10 mg/kg, ip), or Vehicle (Saline with 5% Ethanol, 5% Emulphor)

	spectral counts[a]						
	control (C)		URB597		LY2183240 (L)		relative activity
	ave	SE	ave	SE	ave	SE	L/C
FAAH[b]	23	2	0	0	0	0	0
Abh6[c]	55	3	62	10	3	2	0.06
proteasome β1[d]	11	3	9	2	2	1	0.18
PLA2 group VII[e]	12	4	20	5	3	3	0.25
Wbscr21[f]	19	6	32	10	6	6	0.32
MAG lipase[g]	73	14	120	15	27	8	0.37

[a] Serine hydrolase activities were measured by ABPP-MudPIT using spectral counting, as described previously.[35] Only those hydrolases with at least 10 spectral counts in control proteomes were considered candidates for inhibition by LY2183240 or URB597. Ave = average of 3 independent experiments/group. SE = standard error. For a full list of serine hydrolases identified by ABPP-MudPIT, see Supporting Information Table 1. [b] Fatty acid amide hydrolase. [c] α/β-Hydrolase domain containing 6. [d] Proteasome subunit β-type 1. [e] Phospholipase A2 group VII. [f] Williams−Beuren syndrome critical region protein 21. [g] Monoacylglycerol lipase.

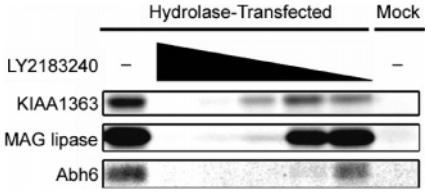

Figure 5. Inactivation of recombinantly expressed serine hydrolases by LY2183240. Two in vivo targets (Abh6 and MAG lipase) and one in vitro target (KIAA1363) of LY2183240 were recombinantly expressed in COS-7 cells and shown by competitive ABPP to be inactivated by LY2183240 (see Table 2 for a list of calculated IC_{50} values). For MAG lipase and KIAA1363, 5000−5 nM LY2183240 (represented in the figure by a descending slope) and 0.6 and 0.2 mg/mL of transfected cell proteome were used in the assays; for Abh6, which proved to be the most potently inhibited target, 50−0.1 nM LY2183240 and 0.01 mg/mL of transfected cell proteome were used to avoid enzyme titration.

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Table 2. IC_{50} Values for the Inhibition of Serine Hydrolases by LY2183240 as Measured by Competitive ABPP[a]

enzyme	source	IC_{50} (nM)	95% confidence
FAAH	brain membrane	13	8.0−20
MAG lipase	COS-7 transfection	5.3	3.9−7.3
Abh6	COS-7 transfection	0.09	0.07−0.10
KIAA1363[b]	COS-7 transfection	8.2	6.1−11
	(brain membrane)	(12)	(7.4−20)

[a] Note that because LY2183240 appears to act as an irreversible inhibitor, the reported IC_{50} values are dependent on the pre-incubation time of the assay (10 min). [b] IC_{50} values are shown for KIAA1363 in both transfected and native (brain membrane) proteomes.

inactivated this enzyme in vitro with an IC_{50} value similar to those observed for MAG lipase and FAAH (Table 2). Regardless, these data underscore the value of profiling inhibitors in living systems, where potential issues such as the cellular and subcellular distribution of these pharmacological agents, as well as their metabolism, are taken into account.

Discussion

The recent description of LY2183240 as a highly potent small-molecule inhibitor of anandamide transport has sparked renewed interest in elucidating the protein(s) responsible for this process.[24,37] Here, we show that LY2183240 is a covalent inhibitor of FAAH, thereby adding to a growing body of evidence that ascribes a primary role for this degradative enzyme in the regulation of anandamide uptake. FAAH's contribution to the cellular uptake of anandamide was originally delineated by Deutsch and colleagues, who showed that FAAH inhibitors reduce this process.[22] More recent studies have revealed that most, if not all, well-characterized anandamide transport blockers inhibit FAAH to a degree that correlates with their impact on anandamide uptake.[19,21] Additionally, the genetic disruption of FAAH from neurons slows anandamide uptake,[16] and conversely, the introduction of FAAH into cells lacking this enzyme increases the rate of this process.[22−24] These findings can be explained by a model in which the rapid intracellular hydrolysis of anandamide by FAAH establishes a concentration gradient that drives the uptake of this lipid.[22,21]

The participation of FAAH in anandamide uptake could also rationalize, at least in part, the saturability of this process, especially at later time points where catabolism might make its greatest contribution. Indeed, careful characterization of the uptake process for anandamide at early time points (less than 1 min) indicates that it is neither saturable[18,21,38] nor sensitive to chemical inhibition.[18,21] These results suggest that the FAAH-independent portion of anandamide uptake into cells may occur largely by passive diffusion. Still, none of these previous studies can exclude the participation of additional proteins, including a putative plasma membrane and/or intracellular transporter, in the cellular uptake and distribution of anandamide, which is supported by some evidence (e.g., partial pharmacological blockade of anandamide uptake in cells lacking FAAH[16,23,39]). They do, however, emphasize that the discovery of such an "anandamide transporter" will require chemical probes that selectively disrupt its function without impacting the activity of FAAH. Our results argue that LY2183240 does not qualify as such an agent, as this compound inhibits FAAH in vitro and in vivo at pharmacologically active doses.

LY2183240 proved to be a remarkably potent inactivator of FAAH (IC_{50} = 13 nM), initially suggesting that it might serve as a useful pharmacological tool for studying this enzyme. However, competitive ABPP studies revealed that LY2183240 also inhibited several other brain serine hydrolases with IC_{50} values in the low nanomolar range. Additional in vivo targets of LY2183240 included lipid-metabolizing enzymes, such as MAG lipase, which has been proposed to regulate the endocannabinoid 2-arachidonoyl glycerol,[40] and uncharacterized hydrolases such as Abh6. Assuming that LY2183240 inactivates each of these enzymes by the same mechanism as FAAH (i.e., carbamylation of the serine nucleophile), these data suggest that the heterocyclic urea group may display excessive inherent reactivity for use in the design of selective pharmacological agents.

The broad target selectivity of LY2183240 might also explain the previous finding that this agent binds to cells that lack FAAH.[24] Perhaps other serine hydrolases sensitive to LY2183240 inactivation are responsible for these FAAH-independent binding events. More generally, our competitive ABPP studies of LY2183240 underscore the importance of defining proteome-wide selectivity patterns for bioactive small molecules to illuminate potentially unanticipated targets of these agents. Indeed, none of the additional enzymes targeted by LY2183240 display any discernible sequence homology with FAAH, suggesting that their common inhibitor sensitivity profiles derive from a higher-order relatedness in active-site structure and/or reactivity. These data thus provide another salient example of enzymes that share "active-site homology" in the absence of significant sequence similarity.[33,41,42]

Experimental Procedures

Synthesis of LY2183240. To a round-bottom flask fitted with a magnetic stirrer were added TBAF·$3H_2O$ (315 mg, 1 mmol, 1 equiv), 4-biphenylacetonitrile (193 mg, 1 mmol, 1 equiv), and trimethylsilyl azide (175 mg, 1.5 mmol, 1.5 equiv). The mixture was heated at 85 °C for 18 h and then cooled to room temperature. The mixture was then diluted with EtOAc (20 mL) and washed with 1 M HCl (3 × 5 mL). The organic layer was then dried with sodium sulfate and concentrated under reduced pressure to afford 5-biphenyl-1-*H*-tetrazole as a white solid (156 mg, 66% yield). The 5-biphenyl-1-*H*-tetrazole (23.6 mg, 0.1 mmol, 1 equiv) was then combined with dimethylcarbamyl chloride (21.4 mg, 0.2 mmol, 2 equiv) and 1,4-diazabicyclo[2.2.2]octane (33.6 mg, 0.3 mmol, 3 equiv) in DMF. After 14 h, the reaction mixture was dried and purified by silica gel chromatography (50% EtOAc in hexanes) to afford LY2183240 (24 mg, 77%). ^{1}H NMR (400 MHz, $CDCl_3$) 7.58−7.54 (m, 4H), 7.44−7.34 (m, 5H), 4.48 and 4.37 (s, 2H), 3.25 and 3.06 (s, 3H), 3.11 and 2.7 (s, 3H); HRMS *m*/*z* calculated for $C_{17}H_{17}N_5O$ $[M + Na]^+$ 330.1325, found 330.1331, 1.8 ppm.

Preparation of Mouse Tissue Proteomes. Mouse tissues were Dounce-homogenized in 50 mM Tris·HCl, pH 8.0, for assays with ^{14}C-oleamide or in 10 mM sodium/potassium phosphate buffer (pH 8) (PB) for ABPP reactions followed by a low-speed spin (1400*g*, 3 min) to remove debris. The supernatant was then subjected to centrifugation

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at 145000*g* for 45 min to provide the cytosolic fraction in the supernatant and the membrane fraction as a pellet that was washed and resuspended in Tris or PB buffer by sonication. Total protein concentration in each fraction was determined using a protein assay kit (Bio-Rad). Samples were stored at −80 °C until use.

In Vitro Analysis of Inhibitor Potency. Inhibitor analysis was carried out in vitro using two different assays. In the first assay, FAAH activity was measured in mouse brain membranes in the presence of varying concentrations of inhibitor by following the conversion of ^{14}C-oleamide, synthesized as previously described,[7] to oleic acid by thin-layer chromatography (TLC).[43] Brain membranes (1 mg/mL) from FAAH (−/−) mice were used as a control. Briefly, varying concentrations of LY2183240 (1 μL, 100× stock in DMSO added to provide 0.001−10 μM final concentration) were preincubated with brain membranes (1 mg/mL in 50 mM Tris, pH 8, 94 μL) for 10 min. ^{14}C-Oleamide (5 μL, 2 mM stock in DMSO, 100 μM final concentration) was added, and 31 μL of each reaction was removed and quenched in 300 μL of 0.1 M HCl at 30, 45, and 60 min. The solution was then extracted with 400 μL of ethyl acetate. The organic layer was removed and dried under a stream of gaseous N_2, solubilized in 25 μL of ethyl acetate, and separated by TLC (60% ethyl acetate in hexanes). The radioactive compounds were quantified using a Cyclone Phosphorimager (PerkinElmer Life Sciences). Inhibitor potency was also examined using competitive ABPP as described previously.[33] Briefly, mouse brain membrane proteomes were diluted to 1 mg/mL in PBS and preincubated with varying concentrations of inhibitors (1 nM to 10 μM, 50× DMSO stocks) for 10 min at room temperature prior to the addition of a rhodamine-tagged fluorophosphonate (FP-rhodamine,[26,27] 50× DMSO stock). The ABPP probe was used at a final concentration of 100 nM in a 50 μL of total reaction volume. Reactions were quenched after 10 min with 2× SDS-PAGE loading buffer, subjected to SDS-PAGE, and visualized in-gel using a flatbed fluorescence scanner. Dose response curves obtained from both methods from three trials at each inhibitor concentration were fit using Prism software (GraphPad) to obtain IC_{50} values with 95% confidence intervals.

MS Analysis of LY2183240 Covalent Labeling of FAAH. Inhibitor labeling reactions were conducted at room temperature for 1 h with 23 μM recombinant purified FAAH (∼74 μg) prepared as described previously,[43] and 35 μM LY2183240 or DMSO for the control reaction in 125 mM Tris·HCl, pH 9, in a total volume of 50 μL. Any noncovalent interactions between inhibitor and enzyme were disrupted by gel filtration through a NAP 5 column (GE Healthcare), the desired fractions were concentrated to dryness by speed vacuum centrifugation, and reconstituted in 0.5% trifluoroacetic acid in H_2O in a final volume of 50 μL. Samples were prepared for MALDI-TOF Reflectron mass spectrometry analysis as previously described with 1:1 α-cyano-4-hydroxycinnamic acid/sample (2 μL of final volume).[30]

In Vivo Labeling of Proteins by LY2183240 in Mice. Mice were male wild type or FAAH (−/−)[9] animals on a C57Bl/6 background, weighed between 20 and 30 g, and were between the ages of 8 and 9 weeks. Mice were given intraperitoneal injections of 10 mg/kg URB597 (in vehicle 18:1:1 saline/emulphor/EtOH), 10 mg/kg LY2183240 (in vehicle), or vehicle alone. After 90 min, the mice were sacrificed by CO_2 asphyxiation and relevant tissues were flash frozen (liquid N_2) immediately upon removal. Tissues were processed as described above to isolate membrane and cytosolic proteomes. The FP-rhodamine labeling was carried out as described above, and reactions were analyzed by SDS-PAGE. See the next paragraph for ABPP-MudPIT analysis of inhibited enzymes in the proteome. The average of three independent samples at each concentration was reported.

ABPP-MudPIT Analysis of Proteins Labeled by LY2183240 in Vivo. Brain membrane proteomes (1 mL, 1 mg/mL in PB) from mice treated with LY2183240, URB597, or vehicle (as described above) were labeled with 5 μM FP-biotin probe for 2 h at room temperature and prepared for MudPIT analysis as previously described except that the Lys-C digestion step was omitted.[34] MudPIT analysis of eluted peptides was carried out as previously described[35,44] on a coupled Agilent 1100 LC-ThermoFinnigan LTQ MS system. All data sets were searched against the mouse IPI database using the SEQUEST search algorithm,[45] and results were filtered and grouped with DTASELECT.[46] Peptides with cross-correlation scores greater than 1.8 (+1), 2.5 (+2), 3.5 (+3) and delta CN scores greater than 0.08 were included in the spectral counting analysis. Only proteins for which 10 or more peptides were identified on average in the control samples were considered for analysis. Specifically, probe-labeled proteins were further identified by their presence in FP-treated samples with a spectral number at least 5-fold or greater than that observed in "no probe" control runs (experiments performed as described above, but without inclusion of biotinylated FP). Spectral counts are corrected for the background observed in the no-probe control and reported as the average of three samples with the standard error (SE) of the mean.

Inactivation of Recombinant Serine Hydrolases by LY2183240. Candidate serine hydrolase targets of LY2183240 were recombinantly expressed in COS-7 cells by transient transfection. COS-7 cells were grown in 100-mm dishes in complete medium (DMEM with L-glutamine, nonessential amino acids, sodium pyruvate, and FBS). The cells were transiently transfected using the appropriate cDNA in the eukaryotic expression vector pcDNA3 (Invitrogen) and the FuGENE 6 transfection system (Roche) following the manufacturer's protocol. Control COS-7 cells were prepared in the same way except that an empty pcDNA3 vector was used for transfection. Cells were washed three times with PBS, collected by scraping, brought up in 400 μL of PBS, and homogenized by sonication, and the membrane fractions were isolated by centrifugation at 145000*g*. Membrane proteomes of KIAA1363-transfected cells and MAG lipase-transfected cells were diluted to 0.2 and 0.6 mg/mL, respectively, in PBS (∼1 nM active enzyme as estimated by ABPP as previously described[27]), and the membrane proteome from Abh6-transfected cells was diluted to 0.01 mg/mL (∼0.1 nM active enzyme). IC_{50} values were obtained by competitive ABPP with FP-rhodamine as described above for FAAH in tissue samples.

Acknowledgment. We thank A. Saghatelian for the synthesis of LY2183240, K. Chiang for assistance with the in vivo mouse studies, S. Niessen for assistance with tandem MS analysis, M. McKinney and K. Masuda for purified recombinant ΔTM-FAAH, G. Siuzdak for access to the MALDI-TOF Reflectron mass spectrometer, and the Cravatt lab for helpful discussions. This work was supported by the National Institutes of Health Grants DA015197 and DA017259 (B.F.C.), the Ruth L. Kirschstein NRSA Postdoctoral Fellowship DA019347 (J.P.A.), the Skaggs Institute for Chemical Biology, and the Helen L. Dorris Institute for the Study of Neurological and Psychiatric Disorders of Children and Adolescents.

Supporting Information Available: Complete ref 6 and Supporting Information Table 1. This material is available free of charge via the Internet at http://pubs.acs.org

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