

BIOCHEMISTRY

Activity-based protein profiling reveals off-target proteins of the FAAH inhibitor BIA 10-2474

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A recent phase 1 trial of the fatty acid amide hydrolase (FAAH) inhibitor BIA 10-2474 led to the death of one volunteer and produced mild-to-severe neurological symptoms in four others. Although the cause of the clinical neurotoxicity is unknown, it has been postulated, given the clinical safety profile of other tested FAAH inhibitors, that off-target activities of BIA 10-2474 may have played a role. Here we use activity-based proteomic methods to determine the protein interaction landscape of BIA 10-2474 in human cells and tissues. This analysis revealed that the drug inhibits several lipases that are not targeted by PF04457845, a highly selective and clinically tested FAAH inhibitor. BIA 10-2474, but not PF04457845, produced substantial alterations in lipid networks in human cortical neurons, suggesting that promiscuous lipase inhibitors have the potential to cause metabolic dysregulation in the nervous system.

In January 2016, a first-in-human study of the fatty acid amide hydrolase (FAAH) inhibitor BIA 10-2474 led to the death of one volunteer and the hospitalization of four others (1–4). All patients manifested mild-to-severe neurological symptoms (3). FAAH is a membrane-bound serine hydrolase that degrades the endocannabinoid anandamide and related amidated lipids (5–8). Three explanations for the clinical neurotoxicity of BIA 10-2474 have been proposed: (i) Errors may have occurred in the clinical trial itself, either in the manufacturing or handling of the compound or in the conduct of the trial; (ii) through its inhibitory effects on FAAH, BIA 10-2474 may have produced high levels of long-chain fatty acid amides (e.g., anandamide) and their oxygenated metabolites, which could potentially overstimulate cannabinoid type 1 (CB₁) receptors (8), transient receptor potential vanilloid 1 (TRPV1) ion channels (9), and/or *N*-methyl-D-aspartate (NMDA) receptors (10); or (iii) BIA 10-2474 and/or its metabolites might have off-target activities. The first hypothesis was dismissed by the French authorities (4). The second hypothesis is considered unlikely because other FAAH inhibitors, such as PF04457845, have exhibited favorable safety profiles in

phase 1 and 2 clinical trials (11, 12). The third hypothesis has not been directly evaluated, because little or no information is available regarding the protein interaction profile of BIA 10-2474 (1).

BIA 10-2474 (Fig. 1A) contains an electrophilic imidazole urea that may react with the nucleophilic serine of FAAH and other serine hydrolases to form covalent and irreversible adducts. We predicted that the serine hydrolase targets of BIA 10-2474 could be identified by using chemical proteomic methods (13–15); this would allow us to compare its selectivity profile to that of PF04457845 (Fig. 1A), a FAAH inhibitor that progressed to phase 2 trials without serious adverse events (11, 12, 16). We first synthesized BIA 10-2474 and BIA 10-2639, a confirmed metabolite in which the *N*-oxide of BIA 10-2474 has been reduced to a pyridine (4) (Fig. 1A), in two independent labs and confirmed their structures by ¹H- and ¹³C-nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (17). Both independently generated sets of compounds displayed equivalent activities in the subsequently described biological assays.

Our initial experiments performed with substrate hydrolysis assays revealed that BIA 10-2474

showed weak in vitro inhibitory activity against human and rat FAAH, displaying median inhibitory concentration (IC₅₀) values ≥1 μM (Fig. 1B, fig. S1, and table S1). Consistent with previous reports (16), PF04457845 potently inhibited FAAH with IC₅₀ values of ~1 to 10 nM (Fig. 1B and table S1). By contrast, BIA 10-2474 exhibited greatly improved potency in cellular assays (in situ), blocking human FAAH activity in transfected HEK293T (human embryonic kidney 293T) cells with IC₅₀ values of 0.05 to 0.07 μM (Fig. 1B). BIA 10-2474 and PF04457845 did not interact with other proteins of the endocannabinoid system or with the endocannabinoid-binding transient receptor potential (TRP) ion channels (tables S2 and S3).

We also created alkynylated analogs of BIA 10-2474—AJ167, AJ179, and AJ198—and found that two of these compounds (AJ179 and AJ198) labeled mouse and human FAAH in brain lysates, as detected by coupling to azide fluorescent reporter groups via copper(I)-catalyzed azide-alkyne cycloaddition (“click”) chemistry (fig. S2) (18). This finding, coupled with the time-dependent inhibition of FAAH displayed by BIA 10-2474 (table S1), provides strong evidence that BIA 10-2474 and related imidazole ureas exhibit an irreversible mode of action.

To investigate the serine hydrolase interaction landscape of BIA 10-2474, we used activity-based protein profiling (ABPP), a chemical proteomic method that uses active site-directed chemical probes [e.g., fluorophosphonates (FPs) or β-lactones for serine hydrolases] to assess the functional state of entire enzyme classes directly in native biological systems (13–15). When coupled to fluorescent reporter groups, ABPP probes enable visualization of enzyme activities in complex proteomes by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and in-gel fluorescence scanning. When coupled to a biotin reporter group, ABPP probes enable affinity enrichment and identification of enzyme activities by mass spectrometry (MS)-based proteomics. In both formats, ABPP serves as a versatile method to assess target engagement and proteome-wide selectivity for small-molecule inhibitors. Gel-based ABPP with a fluorescent FP probe (FP-TAMRA) confirmed the relative in vitro and in situ potencies of BIA 10-2474 and PF04457845 for human FAAH in transfected HEK293T cell preparations (Fig. 1C). The reason for the increased cellular activity of BIA 10-2474 is unclear but could reflect cellular accumulation of the compound, which has been observed for other types of enzyme inhibitors (19).

Initial ABPP studies were performed in the human colon carcinoma cell line SW620, which

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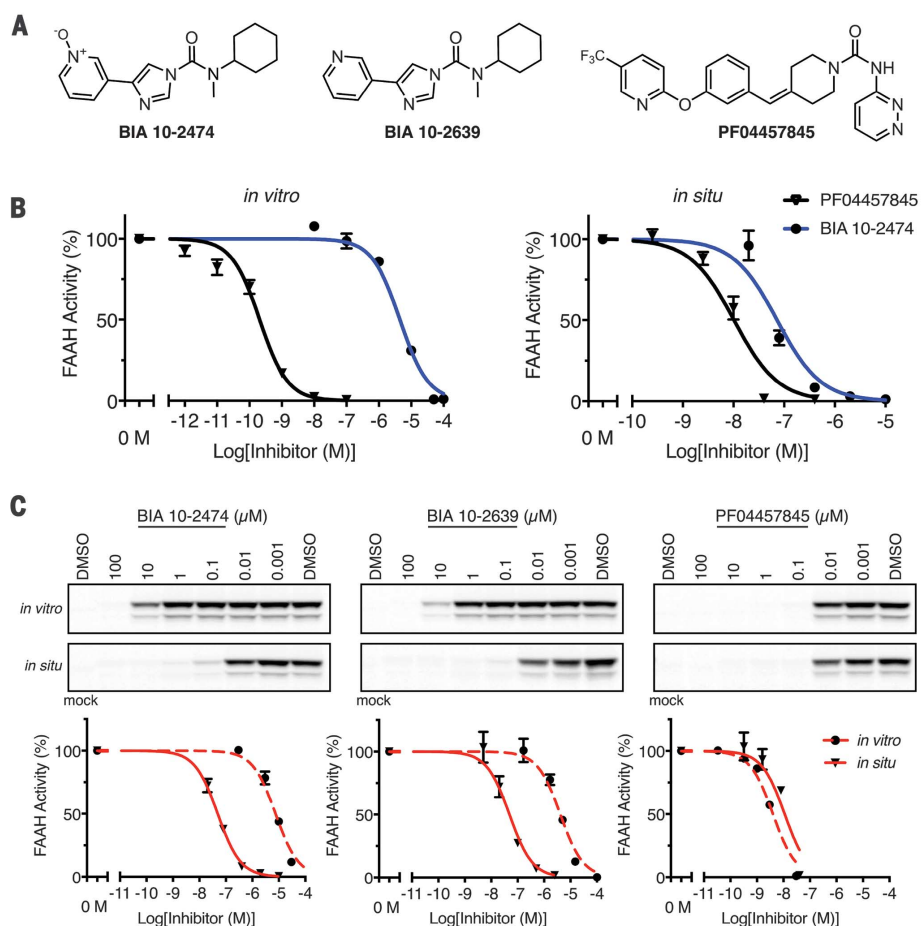


Fig. 1. Comparison of human FAAH inhibition by BIA 10-2474, BIA 10-2639, and PF04457845.

(A) The structures of BIA 10-2474, the metabolite BIA 10-2639, and PF04457845. (B) Inhibition of human FAAH in HEK293T cell lysates (*in vitro*) or intact cells (*in situ*) as measured with an anandamide substrate hydrolysis assay. (C) *In vitro* and *in situ* inhibition of human FAAH as measured by competitive gel-based ABPP. Top: HEK293T cell lysates (*in vitro*) or intact cells (*in situ*) recombinantly expressing human FAAH were pretreated with different amounts of compound or DMSO (*in vitro*: 30 min, 37°C; *in situ*: 4 hours, 37°C). FAAH activity was measured by reactivity with the serine hydrolase-directed probe FP-TAMRA and visualization of signals by gel-based ABPP. Mock indicates control cells transfected with empty vector. Bottom: Corresponding IC_{50} curves for gel-based ABPP data shown above. $N = 3$ independent experiments per group.

Table 1. *In vitro* and *in situ* inhibitory potencies of BIA 10-2474, BIA 10-2639, and PF04457845 against FAAH and representative off-targets. Measurements were made by gel-based ABPP of HEK293T cells recombinantly expressing the indicated human serine hydrolases. Data represent inhibitor treatment of cells for 4 hours with the exception of PNPLA6, where data represent inhibitor treatment of cells for 24 hours. Data represent average values from three independent experiments per group. See table S4 for error measurements related to IC_{50} values. ND, not determined.

Enzyme	Treatment	IC_{50} (μ M)		
		BIA 10-2474	BIA 10-2639	PF04457845
FAAH	<i>In vitro</i>	7.5	4.1	0.004
FAAH	<i>In situ</i> (4 hours)	0.049	0.049	0.011
FAAH2	<i>In situ</i> (4 hours)	0.40	0.10	0.59
ABHD6	<i>In situ</i> (4 hours)	0.081	0.079	>10
CES2	<i>In situ</i> (4 hours)	2.0	0.63	>10
ABHD11	<i>In situ</i> (4 hours)	>10	2.3	>10
PNPLA6	<i>In situ</i> (24 hours)	11	ND	>50

expresses a wide diversity of endogenous serine hydrolase activities, including FAAH and FAAH2. Isotopically heavy and light amino acid-labeled SW620 cells were treated with dimethyl sulfoxide (DMSO) or drug (BIA 10-2474 or PF04457845; 0.2 or 10 μ M each for 4 hours, or 50 μ M each for 24 hours) and then lysed and treated with a biotinylated FP probe. The samples were then combined and subjected to streptavidin enrichment and quantitative liquid chromatography (LC)-MS analysis, in which proteins displaying heavy:light ratios of >2.0 were designated as drug-inhibited targets. We focused on human cell studies and tested a broad range of inhibitor concentrations because the deleterious neurological effects of BIA 10-2474 were observed in humans, but not other mammals, and occurred at drug doses that were 10 to 50 times higher than that required for blockade of FAAH activity in the clinical trial participants (4).

Our MS-based ABPP studies confirmed that both BIA 10-2474 and PF04457845 fully engaged human FAAH at all tested concentrations (0.2, 10, and 50 μ M) (Fig. 2, A and B, and fig. S3). Both drugs showed good selectivity for FAAH at the lowest concentration tested (0.2 μ M; fig. S3). PF04457845 maintained this selectivity profile at higher concentrations, displaying only a single major off-target—the homologous enzyme FAAH2—among ~60 quantified serine hydrolases, consistent with previous studies (16). By contrast, BIA 10-2474 and its metabolite BIA 10-2639 exhibited numerous off-targets across the tested drug concentration range, including FAAH2 and several lipid hydrolases, such as ABHD6, ABHD11, LIPE, and PNPLA6, and xenobiotic drug-metabolizing enzymes CES1, CES2, and CES3 (Fig. 2, A and B). Some of these off-targets, such as ABHD6 and CES2, were almost completely inhibited (>90%) at both 10 and 50 μ M concentrations of BIA 10-2474.

Representative off-targets of BIA 10-2474 were recombinantly expressed in HEK293T cells and verified to engage BIA 10-2474 by gel-based ABPP (Fig. 2C). These experiments also confirmed the relative potency of off-targets mapped by MS-based ABPP, with BIA 10-2474 exhibiting greater inhibitory activity against ABHD6 and CES2 compared to PNPLA6. By contrast, none of the recombinantly expressed enzymes were inhibited by PF04457845 except FAAH2 (Fig. 2C).

Our chemical proteomic data, taken together, demonstrated that both BIA 10-2474 and its major metabolite BIA 10-2639 cross-react with several human serine hydrolases that do not interact with PF04457845 (Table 1 and table S4). One possible contributing factor to this broader interaction profile is the greater intrinsic reactivity of BIA 10-2474 compared to PF04457845, which is reflected in their respective rates of methanolysis (fig. S4). We also note that human CES2 and ABHD6 were both more potently inhibited by BIA 10-2474 and BIA 10-2639 compared to the mouse orthologs of these enzymes (table S2), indicating the potential for species differences in the off-target-mediated activities of these compounds.

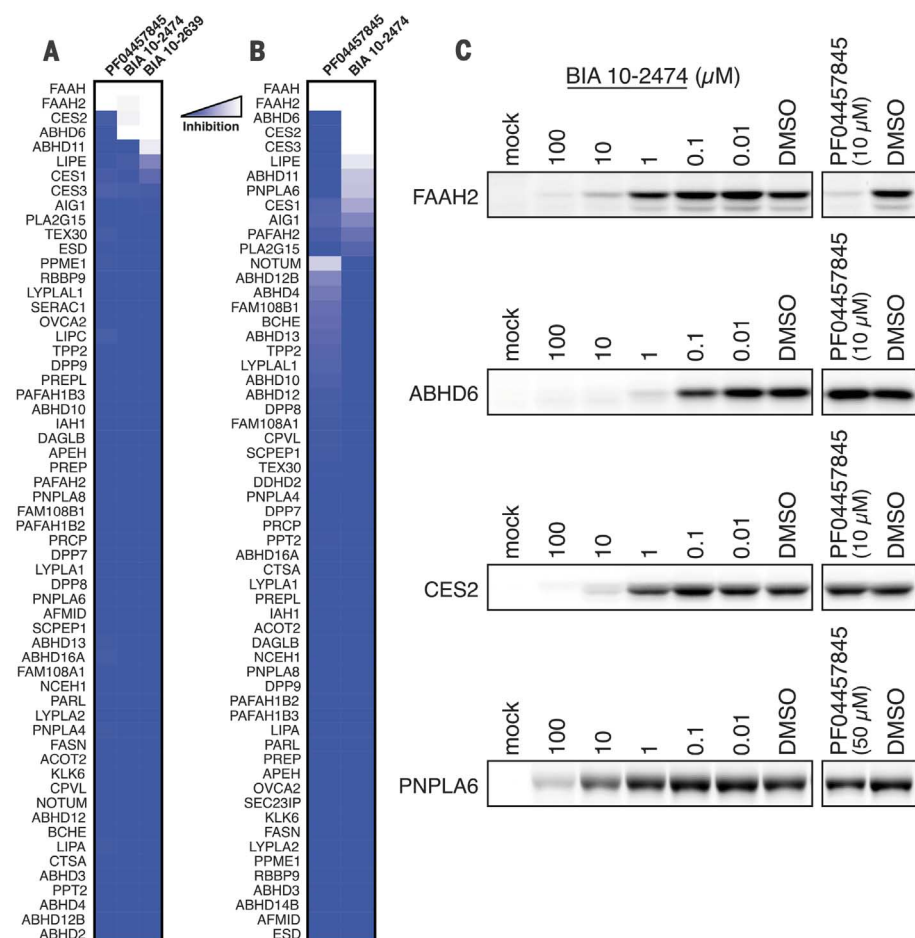


Fig. 2. Quantitative proteomic analysis of serine hydrolase targets of FAAH inhibitors in human cells. (A and B) MS-based ABPP of serine hydrolase activities in SW620 cells treated with DMSO or FAAH inhibitor. Shown in (A) are BIA 10-2474, BIA 10-2639, and PF04457845 (10 μ M, 4 hours, 37°C). Shown in (B) are BIA 10-2474 and PF04457845 (50 μ M, 24 hours, 37°C). Data are expressed as median stable isotope labeling by amino acids in cell culture (SILAC) ratio values for all isotopic peptide pairs quantified per protein from two biological replicates. (C) Confirmation of representative off-targets of BIA 10-2474 by gel-based ABPP of recombinantly expressed enzymes in HEK293T cells.

Many of the off-targets of BIA 10-2474 are involved in cellular lipid metabolism (20, 21), and most (with the exception of FAAH2) show substantial expression in human brain tissue (fig. S5). Although the poor *in vitro* activity displayed by BIA 10-2474 limited our ability to identify off-targets in brain tissue lysates, we could confirm cross-reactivity of this drug with both FAAH and ABHD6 in human frontal cortex proteome (post-mortem samples acquired from three male donors who were 49, 50, and 80 years of age and who were not associated with the BIA 10-2474 trial) (fig. S6). We also observed several of the off-targets of BIA 10-2474 by ABPP of human cortical neurons derived from induced pluripotent stem cells (fig. S7).

We next tested whether prolonged exposure to BIA 10-2474 altered lipid metabolism in human cortical neurons. We performed targeted lipidomic analysis of human cortical neuron cultures treated with vehicle (DMSO) or BIA 10-2474 (50 μ M) at a concentration that was ~20

times greater than the maximum concentration (C_{max}) observed in the human clinical trial (22). In total, the amounts of 161 lipid species were quantified, of which 54 showed a fold change of ≥ 1.20 or ≤ 0.80 when using a Benjamini-Hochberg false-discovery rate $\leq 25\%$ (Fig. 3A and table S5). The lipids affected by BIA 10-2474 included FAAH substrates (*N*-acylethanolamines), as well as several other lipid classes, including triglycerides, monoacylglycerols, (lyso)phosphatidylcholines, free fatty acids, and plasmalogens. By contrast, treatment of human cortical neuron cultures with PF04457845 (1 μ M), which was also tested at a concentration that was 20 times greater than the clinical C_{max} for the drug (17), produced a more restricted profile of lipid changes, predominantly corresponding to the expected elevations of *N*-acylethanolamines (Fig. 3B).

Here we have shown that BIA 10-2474 acts as an irreversible inhibitor of FAAH that displays greater cross-reactivity with human serine hydrolases than the clinically tested FAAH inhibitor

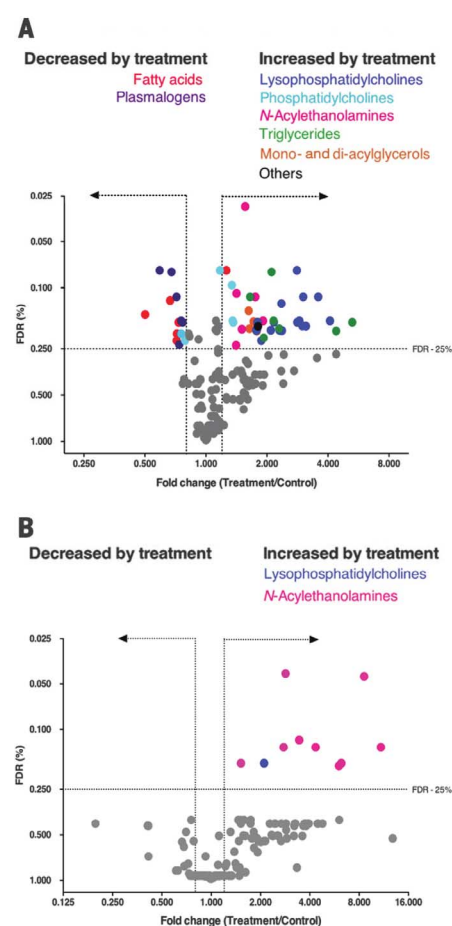


Fig. 3. BIA 10-2474, but not PF04457845, causes substantial alterations in lipid metabolism in human cortical neurons.

(A and B) Cortical neurons were treated with DMSO or BIA 10-2474 (50 μ M) (A) or with DMSO or PF04457845 (1 μ M) (B) and analyzed by MS-based lipidomics after 48 hours. The x axis shows the fold change of lipid species in the inhibitor-treated versus DMSO-treated cells. Lipidomic data are presented as a volcano plot, and lipids with a fold-change threshold of ≥ 1.20 or ≤ 0.80 and a Benjamini-Hochberg false-discovery rate (FDR) $\leq 25\%$ are represented by colored circles indicating lipid class. Data represent average values from at least two independent experiments.

PF04457845. Many of the off-targets of BIA 10-2474 are lipolytic enzymes, raising the possibility that disruption of cellular lipid networks may have contributed to the compound's neurotoxicity. Notably, disruption of neuronal lipid metabolism by inhibition of PNPLA6, one of the off-target proteins of BIA 10-2474 identified in this study, has previously been linked to organophosphate-based neurotoxicity in humans (21, 23–25), and recessive loss-of-function mutations in the *PNPLA6* gene are responsible for a broad spectrum of neurodegenerative disorders (26, 27).

Although our data provide information about the selectivity of BIA 10-2474, they do not allow

us to conclude that inhibition of one or more of the identified off-target proteins is responsible for the clinical neurotoxicity caused by this drug. Nor can we exclude the possibility that noncovalent interactions of BIA 10-2474 or its metabolites with other proteins might have contributed to the reported clinical effects (28). Regardless, our study highlights the general utility of ABPP as a versatile chemical proteomic method to assess on-target engagement and off-target activity of covalent drugs to guide therapeutic development.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/356/6342/1084/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S7
Tables S1 to S5
References (29–44)

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A clue to a drug's neurotoxicity?

The drug BIA 10-2474 inhibits fatty acid amide hydrolase (FAAH), a lipase that degrades a specific endocannabinoid. On the basis of this activity, BIA 10-2474 was being developed as a potential treatment for anxiety and pain. In a phase 1 trial of the drug, one subject died, and four others suffered brain damage. As an initial step in investigating whether inhibition of off-target proteins by BIA 10-2474 might contribute to its clinical neurotoxicity, van Esbroeck *et al.* used activity-based proteomic assays to identify proteins targeted by the drug. Studying human cells and brain samples from subjects not associated with the trial, they found that BIA 10-2474 targeted several different lipases in addition to FAAH. It also substantially altered lipid metabolism in cultured neurons.

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