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A global portrait of protein targets of metabolites of the neurotoxic compound BIA 10-2474

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Abstract

Clinical investigation of the fatty acid amide hydrolase (FAAH) inhibitor BIA 10–2474 resulted in serious adverse neurological events. Structurally unrelated FAAH inhibitors tested in humans have not presented safety concerns, suggesting that BIA 10–2474 has off-target activities. A recent activity-based protein profiling (ABPP) study revealed that BIA 10–2474 and one of its major metabolites inhibit multiple members of the serine hydrolase class to which FAAH belongs. Here, we extend these studies by performing a proteome-wide analysis of covalent targets of BIA 10–2474 metabolites. Using alkynylated probes for click chemistry-ABPP of human cells, we show that des-methylated metabolites of BIA 10–2474 covalently modify the conserved catalytic cysteine in aldehyde dehydrogenases, including ALDH2, which has been implicated in protecting the brain from oxidative stress-related damage. These findings indicate that BIA 10–2474 and its metabolites have the potential to inhibit multiple mechanistically distinct enzyme classes involved in nervous system function.

Fatty acid amide hydrolase (FAAH) is an integral membrane enzyme that terminates the signalling function of the fatty acid amide class of signalling lipids¹, including the

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endogenous cannabinoid (endocannabinoid) anandamide². Inhibitors of FAAH have demonstrated anti-hyperalgesic and anxiolytic effects in preclinical animal models^{3–8} and have been advanced into clinical studies for the potential treatment of a range of central nervous system (CNS) disorders^{9–12}. Original clinical-stage FAAH inhibitors were mostly urea or carbamate agents that irreversibly react with the catalytic serine nucleophile of the enzyme^{3, 6–7, 12–13}. Some of these FAAH inhibitors, such as PF-04457845, were optimized for selectivity using the chemical proteomic method activity-based protein profiling (ABPP)^{14–15} to ensure minimal off-target reactivity within the serine hydrolase class and across the broader proteome. Such FAAH inhibitors have exhibited good safety profiles to date in humans^{9–12}.

More recently, a structurally distinct FAAH inhibitor BIA 10–2474 (**1**, Figure 1) was investigated in a Phase 1 clinical trial and found to cause neurotoxicity that led to the death of one human volunteer and the hospitalization of several other subjects^{16–20}. Considering the generally safe profiles displayed by other FAAH inhibitors in human clinical studies, it has been postulated that the neurotoxicity of BIA 10–2474 (**1**) is due to an off-target mechanism(s). A recent ABPP study focused on serine hydrolases identified several targets of BIA 10–2474 (**1**) and its pyridine metabolite BIA 10–2445 (**2**, Figure 1) that are involved in neuronal lipid metabolism²¹. Nonetheless, whether BIA 10–2474 (**1**) and its metabolites may have additional targets outside of the serine hydrolase class remains unclear.

In this study, we have characterized several major metabolites of BIA 10–2474 (**1**), including not only BIA 10–2445 (**2**), but also the demethylation products, **3** and **4** (Figure 1), and synthesized alkynylated analogues of these compounds for evaluation by click chemistry-ABPP in human cells. These chemical proteomic studies revealed that **3** and **4** covalently react with the catalytic cysteine residues of aldehyde dehydrogenase (ALDH) enzymes in human cells, including members of this enzyme class involved in detoxifying reactive lipid species²².

Results and Discussion

We first evaluated the pharmacokinetics of BIA 10–2474 (**1**, 10 mg/kg, oral administration) and its metabolites in rats, where the concentrations of compounds were measured in plasma, brain, and cerebrospinal fluid (CSF) after 1 h dosing (Table 1). Consistent with previous reports¹⁶, BIA 10–2445 (**2**) was observed as a metabolite where the pyridine *N*-oxide of BIA 10–2474 (**1**) had been reduced to pyridine. Additionally, demethylation products, **3** and **4**, were detected in plasma, brain, and CSF at generally lower concentrations, demonstrating that demethylation occurred for both BIA 10–2474 (**1**) and BIA 10–2445 (**2**).

We surmised that the des-methyl metabolites, **3** and **4**, could show different proteomic reactivity compared to BIA 10–2474 (**1**) and BIA 10–2445 (**2**), and we set out to test this hypothesis by synthesizing alkyne-modified analogues of **1–4** (compounds **5–8**, respectively; Figure 1) for use in CuAAC (copper-catalyzed azide-alkyne cycloaddition), or click chemistry²³ (CC)-coupled activity-based protein profiling (CC-ABPP) studies²⁴. CC-ABPP provides a global portrait of proteins that covalently react with an alkynylated small

molecule in biological systems and has been used to characterize the targets and off-targets of drugs²⁵ and their major metabolites²⁶. For clickable probes, **5–8**, we installed the terminal alkyne at the 4 position of the cyclohexyl group, which we expected would minimally impact physicochemical properties and protein interactions compared to the parent compounds **1–4**. We confirmed that compounds **1–8** all inhibited FAAH with generally similar IC₅₀ values between 0.3–4 μ M (Table S1).

We performed initial CC-ABPP experiments in primary rat cortical neurons, which were treated with **5–8** (1 μ M each) for 1 h, lysed, and alkynylated proteins visualized by CuAAC conjugation with a rhodamine-azide (Rh-N₃) reporter group followed by SDS-PAGE and in-gel fluorescence scanning. All four alkyne probes **5–8** reacted with an ~60 kDa protein – matching the molecular mass of FAAH – and these interactions were blocked by pre-treatment with excess parent compounds (**1–4**, respectively; 10 μ M, 1 h) (Figure S1). The probes showed generally low cross-reactivity with other proteins in rat neurons, although **5** and **6** reacted with an ~30 kDa protein that may represent ABHD6 (Figure S1), a known off-target of BIA 10–2474 (**1**) and BIA 10–2445 (**2**)²¹.

We next evaluated the proteomic reactivity of **5–8** in CCF-STTG1 human astrocytoma cells. We did not observe an ~60 kDa protein in these CC-ABPP experiments, possibly indicating that FAAH is not expressed by CCF-STTG1 cells. In contrast, we observed a distinct, ~50–55 kDa protein that cross-reacted with the des-methyl probes, **7** and **8**, but not probes **5** and **6** (Figure 2). These reactions were blocked by pre-treatment with the corresponding des-methyl parent metabolites (**3** and **4**; 10 μ M, 1 h).

We pursued the identification of the 50–55 kDa target(s) of the des-methyl metabolites of BIA 10–2474 by CC-ABPP coupled with quantitative mass spectrometry (MS)²⁵. In brief, CCF-STTG1 cells were grown in isotopically heavy (H) or light (L)-amino acid media and then treated with DMSO or compounds **3** or **4** (10 μ M, 1 h) followed by probes **7** or **8** (1 μ M, 1 h) (Figure 3A). Cells were then lysed, combined in equal protein amounts, subjected to CuAAC chemistry with a biotin-rhodamine-azide tag, and the probe-labeled proteomes enriched by immobilized streptavidin, digested with trypsin, and the resulting peptides analyzed by LC-MS using a Q-Exactive hybrid quadrupole-Orbitrap MS instrument. These experiments identified a set of three aldehyde dehydrogenase (ALDH) enzymes – ALDH2 (H/L ratio of 20), ALDH1B1 (H/L ratios of 5–10), and ALDH9A1 (H/L ratios of 2–4) – that were competitively blocked in enrichment by **3** and **4** (Figure 3B, C). All three of these ALDH enzymes have predicted molecular weights of ~55 kDa, indicating that the single 50–55 kDa probe **7/8**-labeled band in gel-based CC-ABPP experiments likely corresponded to a combination of these ALDH proteins. We confirmed streptavidin enrichment of ALDH2 by probes **7** and **8**, and blockade of this enrichment by **3** and **4**, respectively, using Western blotting (Figure 3D).

ALDHs are cysteine-dependent enzymes and therefore susceptible to covalent reactivity with and inhibition by electrophilic small molecules^{27–30}. We tested whether the des-methyl metabolites of BIA 10–2474 (**1**) reacted with catalytic cysteines in ALDH enzymes using the chemical proteomic method isoTOP-ABPP, which globally quantifies cysteine reactivity using an alkynylated iodoacetamide probe (IA-alkyne)^{31–32} (Figure 3E). In brief, SW620

cells were treated with compound **3** (10 μ M, 1 h) or DMSO and then subject to isoTOP-ABPP, which revealed that, among the > 1500 quantified cysteines, only C319 of ALDH2 and C288 of ALDH9A1 were blocked in their IA-alkyne reactivity by compound **3** (Figure 3F). Both C319 of ALDH2 and C288 of ALDH9A1 are the conserved cysteine nucleophiles of these enzymes. Other cysteines in ALDH2 were quantified by isoTOP-ABPP (C386) and found to be unperturbed by **3** (Figure 3F, inset), indicating that this compound reacts site-specifically with the catalytic nucleophile of ALDH2. We did not detect the catalytic cysteine for the third ALDH target of compound **3** – ALDH1B1 – in our isoTOP-ABPP experiments. Notably, compound **3** did not react with all of the quantified cysteine nucleophiles in ALDH enzymes, as C340 of ALDH5A1 was unchanged in **3**-treated cells (Table S2). And, consistent with the gel-based CC-ABPP data, which showed a single compound **3**-sensitive, probe **7**-labeled protein band (Figure 1), we did not observe any other cysteines that were blocked in IA-alkyne reactivity by compound **3** except the catalytic cysteines of ALDHs (Figure 3F).

We confirmed the reactivity of des-methyl metabolites of BIA 10–2474 (**1**) with C319 of ALDH2 by site-directed mutagenesis. In brief, we treated HEK293T cells recombinantly expressing FLAG epitope-tagged wildtype (WT) or a C319A mutant of ALDH2 with probe **7** (1 μ M, 1 h) and then analyzed the samples by gel-based CC-ABPP, which revealed strong reactivity of WT-ALDH2, but not the C319A mutant with probe **7** (Figure 4A).

We finally pursued structural characterization of the adduct between compound **3** and C319 of ALDH2. HEK293T cells expressing FLAG-tagged WT-ALDH2 were treated with **3** (10 μ M, 1 h), lysed, and immuno-precipitated with anti-FLAG beads. Enriched proteins were then reduced, alkylated, trypsinized, and analyzed by LC-MS-based proteomics. This analysis confirmed the presence of an *S*-thiocarbamate adduct between compound **3** and C319 on ALDH2 (Figure 4B and Figure S3). These results indicate that the des-methyl metabolites carbamylate the nucleophilic cysteine of ALDHs. While this adduct resembles the reaction products of BIA 10–2474 (**1**) and its metabolites with FAAH, the mechanism of modification of ALDHs by **3** and **4** is likely different from the direct carbamylation observed with serine hydrolases. Imidazole NH ureas are often referred to as “blocked isocyanates” because they form isocyanates when heated or exposed to a catalyst³³. Thus, although direct nucleophilic attack of ALDH2 C319 at the NH urea cannot be ruled out, one possibility is that **3** and **4** undergo an enzyme-mediated deblocking reaction to generate isocyanates that then covalently modify the active-site cysteine in ALDHs (Figure 4C). This pathway would not be available to *N*-methyl compounds BIA 10–2474 (**1**) and BIA 10–2445 (**2**), explaining why they fail to react with ALDHs. This proposed mechanism may also explain the selectivity of **3** and **4** for ALDHs relative to other proteins containing reactive cysteine residues. A similar route for covalent inhibition of ALDHs by 3-amino-1-phenyl-1-propanone compounds has been put forward, where a base-catalyzed β -elimination of the amine furnishes a vinyl ketone that modifies the nucleophilic cysteine³⁴.

In summary, we show using ABPP methods that the des-methyl metabolites (**3** and **4**) of the neurotoxic compound BIA 10–2474 (**1**) covalently modify the catalytic cysteine residue of multiple ALDH enzymes in human cells. This interaction is not observed with BIA 10–2474

(1) or alternative metabolites, such as BIA 10–2445 (2), that retain the *N*-methyl imidazole urea group, indicating that loss of the methyl substituent is required for ALDH modification. While we do not know whether the inhibition of ALDHs would occur at toxicologically relevant doses of BIA 10–2474 (1) in humans, we should note that ALDH2, in particular, has been proposed to play an important role in protecting the brain from toxic aldehyde metabolites formed by excessive reactive oxygen species (ROS)^{35–36}. From a methodological perspective, our findings demonstrate how ABPP can be used to assess the full scope of protein reactivity displayed by electrophilic drugs and their metabolites in human cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of BIA 10-2474 (1), metabolites (2-4) and corresponding clickable probes (5-8).

Figure 2.

Gel-based CC-ABPP analysis of CCF-STTG1 astrocytoma cells treated with DMSO (–) or compounds **1–4** (10 μ M, 1 h) followed by the corresponding alkyne probes **5–8** (1 μ M, 1 h). Probe-labeled proteins were visualized by CuAAC to an Rh-N₃ reporter group, SDS-PAGE, an in-gel fluorescence scanning. Fluorescent gel shown in grayscale. See Figure S2 for Coomassie blue staining of the proteomic samples.

Figure 3.

Identification of protein targets of BIA 10-2474 metabolites **3** or **4** by quantitative MS-based CC-ABPP. (A) Schematic for MS-based ABPP experiments. (B, C) Heavy/light isotopic ratios for proteins in CCF-STTG1 cells treated with DMSO (heavy) or BIA 10-2474 metabolites, **3** (B) or **4** (C) (10 μM, 1 h; light), followed by treatment with the corresponding clickable probe, **7** (B) or **8** (C) (1 μM, 1 h), respectively. Data represent average ratio values for quantified peptides for each protein from two independent biological replicates. (D) Anti-ALDH2 Western blot of streptavidin-enriched fractions from CCF-STTG1 cells treated with clickable probes **7** or **8** (1 h, 1 μM), with or without pre-treatment using the corresponding parent compounds (**3** or **4**, 1 h, 10 μM). (E) Schematic for isoTOP-ABPP experiments. (F) isoTOP-ABPP ratios (heavy/light) for quantified cysteines in SW620 cells treated with either compound **3** (10 μM, 1 h; light) or DMSO (heavy). Data represent average values across four independent biological replicates. *Inset*: representative MS1 spectra of the active site cysteine (C319)-containing peptide, and a non-active site cysteine (C389)-containing peptide from ALDH2. Red and blue traces represent inhibitor and DMSO-treated samples, respectively.

Figure 4.

Characterization of C319 as the site of compound 3/probe 7 reactivity in ALDH2 and proposed reaction mechanism. (A) Gel-based ABPP and western blot analysis of HEK-293T cells transiently transfected with cDNAs for FLAG-WT-ALDH2 or FLAG-C319A-ALDH2 (or empty vector; mock), where cells were treated with probe 7 (1 μ M, 1 h), followed by harvesting, lysis, CuAAC conjugation with a rhodamine-azide (Rh-N₃) tag, and SDS-PAGE analysis coupled to in gel-fluorescence scanning. Western blotting was performed with an anti-FLAG antibody. (B) MS1 trace for the m/z ion corresponding to the carbamylated adduct of compound 3 with the active site peptide of ALDH2. HEK-293T cells over-expressing ALDH2 with a C-terminal FLAG tag were treated with compound 3 (*in situ*, 10 μ M, 1 h). See Figure S3 for tandem MS (MS/MS) data supporting assignment of C319 as site of carbamylation by 3. *represents carbamidomethylated cysteines. (C) Proposed mechanism for ALDH2 reaction with compound 3 involving the formation of an isocyanate in the active site.

Table 1.

Plasma, brain, and CSF pharmacokinetic data following administration of BIA 10–2474 (**1**) in rat (10 mg/kg formulated in 0.5% methylcellulose, p.o. dosing). Time of maximal concentration for all analytes was 1 h.

Compound	Plasma		Brain		CSF	
	C _{max} (ng/mL)	AUC _{0–7} (ng·h/mL)	C _{max} (ng/mL)	AUC _{0–7} (ng·h/mL)	C _{max} (ng/mL)	AUC _{0–7} (ng·h/mL)
BIA 10–2474 (1)	4563	13800	939	2480	1300	4000
2	99.2	306	252	185	36.2	113
3	106	323	4.57	11.8	5.03	15.6
4	14.6	50.2	14.4	34.9	1.79	4.91 [*]

AUC_{0–7} = Area under the matrix-time concentration curve from 0–7 hrs; C_{max} = Maximum concentration in matrix; CSF = cerebrospinal fluid. Data not corrected for binding to tissues.

^{*} Represents AUC_{0–4} as samples were below the level of quantitation after 4 post-dose.