Activity-Based Probe for N-Acylethanolamine Acid Amidase

Elisa Romeo,†‡ Stefano Ponzano,*+‡ Andrea Armirotti, ‡ Maria Summa, ‡ Fabio Bertozzi, † Gianpiero Garau, † Tiziano Bandiera, † and Daniele Piomelli*+§

1Drug Discovery and Development, Istituto Italiano di Tecnologia, Via Morego 30, I-16163 Genova, Italy
2Departments of Anatomy and Neurobiology, Pharmacology, and Biological Chemistry, University of California, 3216 Gillespie Neuroscience Facility, Irvine, California 92697-4621, United States

ABSTRACT: N-Acylethanolamine acid amidase (NAAA) is a lysosomal cysteine hydrolase involved in the degradation of saturated and monounsaturated fatty acid ethanolamides (FAEs), a family of endogenous lipid signaling molecules that includes oleoylethanolamide (OEA) and palmitoylethanolamide (PEA). Among the reported NAAA inhibitors, α-amino-β-lactone (3-aminoxetan-2-one) derivatives have been shown to prevent FAE hydrolysis in innate-immune and neural cells and to reduce reactions to inflammatory stimuli. Recently, we disclosed two potent and selective NAAA inhibitors, the compounds ARN077 (5-phenylpentyl-N-[(2S,3R)-2-methyl-4-oxo-oxygen-3-y1]carbamate) and ARN726 (4-cyclohexylbutyl-N-[(S)-2-oxazetidin-3-y1]carbamate). The former is active in vivo by topical administration in rodent models of hyperalgesia and allodynia, while the latter exerts systemic anti-inflammatory effects in mouse models of lung inflammation. In the present study, we designed and validated a derivative of ARN726 as the first activity-based protein profiling (ABPP) probe for the in vivo detection of NAAA. The newly synthesized molecule 1 is an effective in vitro and in vivo click-chemistry activity-based probe (ABP), which is able to capture the catalytically active form of NAAA in Human Embryonic Kidney 293 (HEK293) cells overexpressing human NAAA as well as in rat lung tissue. Competitive ABPP with 1 confirmed that ARN726 and ARN077 inhibit NAAA in vitro and in vivo. Compound 1 is a useful new tool to identify activated NAAA both in vitro and in vivo and to investigate the physiological and pathological roles of this enzyme.

Supporting Information

N-Acylethanolamine acid amidase (NAAA) is a cysteine hydrolase that belongs to the N-terminal nucleophile (Ntn) family of enzymes.1–3 It is localized to lysosomes and bears a significant degree of sequence homology with the bacterial choloylglycine hydrolases, which are characterized by the ability to cleave nonpeptide amide bonds.4 Like other Ntn enzymes, NAAA is activated by autoproteolysis at acidic pH, which generates a catalytically competent form of the enzyme.3 Site-directed mutagenesis experiments have unequivocally identified Cys131 (in mice) or Cys126 (in humans) as the amino acid residue responsible for both systemic anti-inflammatory effects and catalytic activity.

NAAA shares 33–34% identity with acid ceramidase (AC), another cysteine amidase found in lysosomes.1 AC catalyzes the hydrolysis of medium-chain ceramides, which are important mediators of cell senescence and apoptosis9,10 and the formation of sphingosine, while NAAA is involved in the deactivating hydrolysis of fatty acid ethanolamides (FAEs), a class of lipid mediators that play important roles in the control of pain, inflammation, and energy balance.11,12 In particular, NAAA preferentially hydrolizes monounsaturated and saturated FAEs, such as oleoyl ethanolamide (OEA, 18:1 Δ9) and palmitoylethanolamide (PEA, 16:0),1,2,13 over polyunsaturated FAEs like arachidonylethanolamide (anandamide, 20:4 Δ5,8,11,14) which is preferentially inactivated by fatty acid amid hydrolase (FAAH).14,15 Anandamide is an endogenous agonist for cannabinoid receptors and participates in the control of stress-coping responses and pain initiation,16 among other functions, while PEA and OEA regulate pain, inflammation, and energy balance primarily by ligating peroxisome proliferator-activated receptor-α (PPAR-α), a member of the nuclear receptor superfamily.17–19

The pharmacology of PEA has been extensively investigated.15 The compound inhibits peripheral inflammation and mast cell degranulation20,21 and exerts profound antinociceptive effects in rat and mouse models of acute and chronic pain.11,21–25 Moreover, in mice, PEA suppresses pain behaviors induced by tissue injury, nerve damage, or inflammation.18 These effects are dependent on PPAR-α activation, since they are absent in PPAR-α-null mice, blocked by PPAR-α antagonists, and mimicked by PPAR-α agonists.18,24

A limited number of natural25 or synthetic26–31 NAAA inhibitors have been reported thus far. Among them, α-amino-
β-lactone (3-aminooxetan-2-one) derivatives have shown promise because of their high inhibitory potency and target selectivity.32–36

In vitro and in vivo studies have demonstrated that this class of compounds blocks PEA and OEA hydrolysis in host-defense cells, such as macrophages and sensory neurons, and dampens tissue reactions to various inflammatory stimuli.34,35 For example, topical administration of 5-phenylpentyl-N-[(2S,3R)-2-methyl-4-oxo-oxetan-3-yl]carbamate (ARN077, Figure 1),32,33 a potent and selective β-lactone NAAA inhibitor [rat

NAAA (rNAAA) median inhibitory concentration, IC₅₀ = 50 nM, human NAAA (hNAAA) IC₅₀ = 7 nM], elevates PEA and OEA levels in mouse skin and sciatric nerve and attenuates inflammatory effects in mouse models of carrageenan- and LPS-induced inflammation and suppresses inflammatory reactions in human macrophages ex vivo.39 These effects are ascribable to PPAR-α activation by endogenous PEA and OEA, the levels of which are increased after treatment with the inhibitor.39

High-resolution liquid chromatography–tandem mass spectrometry (LC–MS) analysis enabled us to elucidate the mechanism through which β-lactone and β-lactam derivatives inhibit NAAA activity. We showed that ARN077 and ARN726 block NAAA via formation of a thioester bond with its catalytic cysteine.39,40 This result, together with the systemic activity demonstrated by ARN726,39 prompted us to design an activity-based protein profiling (ABPP) probe that might be used to detect catalytically active NAAA in live animals. Similar β-lactams have been used as ABPP probes in bacterial proteomes to identify penicillin-binding proteins and other relevant targets.31,42 Here, we describe a novel β-lactam ABPP probe for NAAA and utilize click chemistry-ABPP (CC-ABPP) methodology33,44 to elucidate the in vitro and in vivo labeling profile of this compound.

The CC-ABPP technique (Figure 2) contemplates the use of a chemical probe with a terminal alkyne functionality, which is first incubated with intact cells or cell extracts and subsequently modified by 1,3-dipolar Huisgen cycloaddition35–48 to introduce a reporter tag (e.g., biotin or rhodamine). Reacted proteins bearing the reporter tag can be subsequently identified by in-gel fluorescence detection, Western blot, or LC–MS analysis (Figure 2). This two-step method allows for a more efficient labeling of reactive proteins compared with the original ABPP procedure, where the reporter tagged probes usually show low cell permeability and lower selectivity toward target enzymes.41,43

RESULTS AND DISCUSSION

The design of the ABPP probe 1 (ARN14686, undec-10-ynyl-N-[(3S)-2-oxoazetidin-3-yl]carbamate) was based on the chemical structure of the systemically active NAAA inhibitor ARN726 (Figure 1).39 We did not modify the azetidin-2-one ring of ARN726, which reacts with the catalytic cysteine of NAAA but replaced the 4-butyl-cyclohexyl group with a C9 saturated aliphatic chain bearing a terminal alkyne tag (Figure 2a). We selected a C9 chain for two reasons: first, previous work had shown that NAAA displays higher affinity for inhibitors with long aliphatic chains,3,38 and second, we expected that such a chain would facilitate the interaction between the alkyne moiety of 1 and the azide group of the reporter tag during the click chemistry reaction. The chemical synthesis of 1 is described in the Supporting Information.

We examined the ability of 1 to inhibit NAAA activity by assessing the hydrolysis of N-(4-methyl-2-oxo-chromen-7-yl)-hexadecanamide (PAMCA) using recombinant hNAAA heterologously expressed in HEK293 cells (see Supporting Information). Compound 1 inhibited hNAAA with high potency (IC₅₀ = 6 nM compared with IC₅₀ = 73 nM for ARN726). The compound also inhibited rNAAA (IC₅₀ = 13 nM), while having only a weak inhibitory effect on rat AC (IC₅₀ = 1.2 μM). As previously shown for ARN726,39 compound 1 interacts with NAAA via covalent binding to the N-terminal cysteine. High-resolution LC–MS analyses showed that probe

![Figure 1](https://example.com/f1.png)  
**Figure 1.** Structures of NAAA inhibitors: β-lactone ARN077 and β-lactam ARN726.

![Figure 2](https://example.com/f2.png)  
**Figure 2.** (a) Structure of the click chemistry probe 1. (b) General strategy for click chemistry activity-based protein profiling (CC-ABPP). Whole cells or lysates are incubated with the probe and subjected to click chemistry to introduce the reporter tag. The labeled proteome is visualized by in-gel or Western blot analysis before and after enrichment on streptavidin beads. Protein identification is carried out by mass spectrometry of the enriched proteome tryptic peptides.
1, upon incubation at acidic pH (4.5) with purified hNAAA, forms a covalent adduct with the N-terminal peptide of the enzyme through S-acylation of its catalytic cysteine. Figure 3 reports the full MS/MS spectrum of hNAAA N-terminal peptide CTSIVAQDSR acylated by 1. Labeling occurs on the cysteine residue (see b fragment ion series reported in Figure 3b) and two side-chain fragments are consistent with the formation of a thioester adduct (Figure 3c).

To evaluate the usefulness of 1 as a click chemistry probe, we incubated the compound with purified hNAAA (which was preventively activated at acidic pH), coupled an azide−PEG3−biotin conjugate to its terminal alkyne using click chemistry, and visualized protein bands using streptavidin−horseradish peroxidase (HRP). The labeling step was performed either in phosphate buffered saline (PBS, pH 7.4) or in phosphate/citrate buffer, pH 4.5, while in both cases PBS was used as buffer for the click chemistry reaction. As shown in Figure 4a, a chemiluminescent signal was visible at the apparent molecular mass of catalytically active NAAAβ-subunit, and no differences were observed at the two chosen pH values. By contrast, both active enzyme and full-length inactive protein were detected when using an anti-NAAA antibody. This result indicates that 1 binds only to the catalytically active form of NAAA and may serve therefore as an efficient activity-based probe. The interaction of 1 with NAAA was further evaluated using different concentrations of the probe with fixed amounts of purified hNAAA, or vice versa. As shown in Figure 4b, when increasing concentrations of 1 (from 0.01 to 10 μM) were incubated with a fixed amount of purified hNAAA (1 μM), a proportional increase in chemiluminescent signal was noted. A similar result was obtained when changing the protein amount while keeping the probe concentration constant (10 μM) (Figure 4c). We performed this experiment in the presence of a background proteome (10 μg of HEK293 cell extract). As shown in Figure 4c, the lowest concentration of NAAA detected by the probe was 1.25 pmol.

We further validated 1 by testing the ability of the probe to label intact HEK293 cells that overexpress hNAAA (NAAA-HEK293). As shown in Figure 5a, incubations of intact cells or cell lysates with 1 yielded results similar to those obtained with purified enzyme, and only bands associated with the activated

---

**Figure 3.** (a) Full MS/MS spectrum of hNAAA peptide CTSIVAQDSR acylated by 1. (b) Zoomed range showing b1, b2, and b3 backbone fragment ions indicating that the acylation occurred on the N-terminal cysteine residue. (c) Further zoom of the same mass range, showing two side-chain fragmentations (F1 and F2) that are diagnostic for a thioester formation (F1 = [C15H25N2O3S]+, expected m/z 313.1586, measured m/z 313.1580, 1.9 ppm mass error; F2 = [C15H25N2O3]+, expected m/z 281.1865, measured m/z 281.1860, 1.8 ppm mass error).

**Figure 4.** Labeling of purified hNAAA. (a) Protein blot analysis of activated recombinant hNAAA incubated with DMSO (−) or compound 1 (+) at pH 4.5 or 7.4. The blotting membranes were probed with streptavidin−HRP conjugate or anti-NAAA antibody (α-NAAA), as indicated. (b) Concentration dependence of the interaction of 1 with NAAA. Compound 1 was incubated at various concentrations with a constant amount of hNAAA (1 μM). (c) Limit of detection of hNAAA by 1. hNAAA was incubated at various concentrations with a constant amount of 1 (10 μM) in the presence of 10 μg of protein extract from HEK293 cells. Blotting membranes in panels b and c were probed with streptavidin−HRP conjugate; FL, full-length protein; β, NAAA β-subunit; Pb = Protein blot; C = Coomassie blue staining.
β-subunit of NAAA were tagged by streptavidin−HRP. When the anti-NAAA antibody was applied to blot membranes both intact and cleaved NAAA were detected, with a prevalence of the activated form of the enzyme. This experiment highlights the high versatility of 1, which can be efficiently used to detect NAAA both in cell lysates and in intact cells where the labeling occurs inside the lysosomes. In a similar experiment (Figure 5b), we preincubated NAAA-HEK293 cells with ARN726 (lane 3) or ARN077 (lane 4) and then added an equimolar concentration of 1. In either case, a decrease in signal intensity was observed, but a more pronounced masking of NAAA was noted with ARN726 than with ARN077. This is consistent with the partial reversibility of ARN077 observed in dialysis experiments and with our findings that the covalent adduct formed by β-lactones with NAAA undergoes hydrolysis under the conditions of the assay, whereas the covalent adduct formed by β-lactams does not (unpublished data).

To check whether the signal of the band associated with overexpressed NAAA could partially or completely mask other unwanted targets, we repeated the experiment using wild-type (wt) HEK293 cells, which do not constitutively express NAAA (Supplementary Figure S1, Supporting Information). The cells were incubated in the presence of 1, and then cytosol and membrane fractions were isolated. The entire CC-ABPP protocol was carried out, including the enrichment phase on streptavidin beads and LC−MS analysis of the enriched proteome. As a positive control, we used a maleimide-containing probe (2, see Supporting Information), incubated it with wt HEK293 cells, and applied the CC-ABPP procedure to cytosol and membrane fractions. As shown in Figure 6a, in wt HEK293 incubated with 1, no significant bands were detected by protein blot. Conversely, when the same cells were incubated with 2, intense chemiluminescent signals were observed throughout the range of molecular masses, indicating that HEK293 cells express a large number of proteins that could potentially react with an electrophilic probe such as 1. The total lysate of NAAA-HEK293 incubated with 1 was used as positive control (lane 5, Figure 6a). To further evaluate the possible presence of off-targets of 1, the same sample from HEK293 or NAAA-HEK293 incubated with 1 was enriched using streptavidin−agarose, and the eluted proteins were analyzed by protein blot. As shown in Figure 5b, no spurious target could be detected in HEK293 cells, even after the enrichment phase, whereas NAAA was clearly identified in HEK293 cells overexpressing the enzyme.
As last step to ensure the identification of all possible targets of I, the streptavidin enriched proteins of HEK293 and NAAA-HEK293 were also analyzed by LC–MS/MS. The findings confirmed that NAAA was the only labeled enzyme in NAAA-HEK293 cells (Supplementary Table S1, Supporting Information).

The ability of I to label NAAA in intact cells encouraged us to examine the efficiency and selectivity of the probe under more challenging experimental conditions, such as those offered by in vivo treatments and analysis of homogenates and tissue fractions prepared from freshly dissected organs. We examined therefore whether I was able to label NAAA in rat lungs, which are known to contain the enzyme in relatively high levels.2 We first prepared various tissue fractions (total lysate, membrane, cytosol, and lysosome fractions) and incubated them with two different concentrations of I (1 and 10 μM). We performed the CC-ABPP protocol, including the streptavidin enrichment phase, and analyzed the labeled proteins by protein blot as shown in Figure 7a. Consistent with our previous findings, a protein band with the apparent molecular mass of NAAA was visualized in the lysosomal fraction incubated with the two different concentrations of I (1 or 10 μM). At the highest concentration used, another weaker signal with apparent molecular mass of 37 kDa was observed. Moreover, a protein band at around 68 kDa was detected in the total lysate and the cytosolic fraction. LC–MS analyses of the streptavidin-enriched proteins confirmed the presence of rNAAA in the lysosome fraction and identified the 37 kDa band as AC, while the 68 kDa protein corresponded to serum albumin. These additional targets were present only in samples exposed to the highest concentration of I, which at 1 μM was highly selective for NAAA (Supplementary Table S2, Supporting Information). The expression of NAAA and AC in the rat lung cellular fractions was analyzed also by Western blot using specific antibodies (Supplementary Figure S2, Supporting Information).

Finally, we investigated the ability of I to act as a selective activity-based probe for NAAA in vivo. We administered the compound by intravenous (iv) injection (10 mg/kg) in either naive rats or rats that were pretreated with ARN726 (10 mg/kg, iv). Lysosomal lung preparations were used for click chemistry and streptavidin enrichment. The protein blot results illustrated in Figure 7b indicate that ARN726 competed with I for binding to both NAAA and AC (lane 3, Figure 7b). The binding of ARN726 and I to AC is not unexpected, given the high exposure achieved with the 10 mg/kg iv dose of the two compounds. In agreement with previous results, when rats were treated only with I the bands associated with NAAA and AC (lane 4, Figure 7b) were clearly visible. The presence of NAAA and AC as the only targets of I was confirmed by LC–MS analysis of the enriched proteome (Supplementary Table S3, Supporting Information).

In conclusion, based on the scaffold of the serine-derived β-lactam ARN726, we designed and validated compound I as the first activity-based probe for NAAA. β-Lactones and β-lactams are known to react with enzymes bearing active site nucleophiles, and thanks to the covalent nature of their inhibition and their customizable reactivity, they may be especially suitable for the design of activity-based probes.31 Since mammals express a substantial number of proteins that could be potentially targeted by β-lactone and β-lactam probes, selectivity is an important issue. In this study, we provide evidence that I efficiently binds to the catalytically active form of NAAA and displays very high target selectivity both in vitro and in vivo. Among hundreds of known serine and cysteine proteases,50 I interacted only with NAAA, indicating that it may offer a useful tool to identify activated NAAA in animal models and other complex biological systems. The recent finding that NAAA may be involved in tumor aggressiveness51 highlights the relevance of this result. Moreover, we showed that, when administered at high concentrations or doses (10 μM in vitro, 10 mg/kg in vivo), I is able to bind AC, a key enzyme of sphingolipid metabolism that shares 33-34% identity and 70% similarity with NAAA. Uracil derivatives have been recently disclosed, which may be useful as ABPP probes for AC.52 Our results suggest that the β-lactam scaffold may offer an additional starting point for the development of activity-based probes for AC and, possibly, other cysteine hydrolases.
Sample Preparation for CC-ABPP Analysis on Purified hNAAA. Purified hNAAA (2 μM) was incubated in activation buffer [100 mM sodium phosphate buffer, 100 mM sodium citrate, 3 mM tris(2-carboxyethyl)phosphine (TCEP) hydrochloride, 0.1% Triton X100, pH 4.5] for 2 h at 37 °C. The labeling with compound 1 (20 μM, for 1.5 h at 37 °C) was performed either in activation buffer (pH 4.5) or in PBS (pH 7.4) following a buffer exchange step using desalting columns. After incubation with the probe, the buffer of both samples was exchanged with PBS, and the click chemistry reaction was performed. Control samples containing DMSO were also prepared. For the concentration-response curves, the amount of NAAs and 1 were as indicated in Figure 4, and the labeling pH was pH 7.4. To determine the detection limit of NAAA by 1 (Figure 4c), the purified protein (5 μg) was dissolved in 100 μl of wt HEK293 protein extract (1 mg mL⁻¹, in PBS buffer), and then 1:2 serial dilutions in HEK293 proteome (1 mg mL⁻¹) were prepared. Protein samples were subjected to click chemistry for biotin addition, as described below, and samples (10 μl) were analyzed by gel-electrophoresis and protein blot.

Sample Preparation for CC-ABPP Analysis on Cell Lines. For the in vitro CC-ABPP on cell lysates, NAAA-overexpressing HEK293 cells (1 × 10⁸) were lysed by sonication (Sonopuls HD 2200, Bandelin, D) in 4 volumes of PBS/sucrose 0.32 M at pH 7.4 and protease inhibitor cocktail (Sigma-Aldrich product #P8340). The suspension was centrifuged at 800 g for 20 min at 4 °C to discard nuclei and cell debris. The total extracted proteins were quantified with BCA protein assay (Euroclone). The protein extracts were diluted to 2 mg mL⁻¹ (500 μL) and incubated with 1 (10 μM) or DMSO only, for 1 h at 37 °C. Click chemistry was performed as described below. For the in-cell CC-ABPP, cells (wild-type HEK293 or hNAAA overexpressing HEK293) were cultured in suspension with FreeStyle 293 expression medium (Life Technologies) and treated with 1 or 2 (40 μM) for 1 h at 37 °C. For competitive ABPP, cells were first incubated with ARN726 (40 μM) or ARN707 (40 μM), and then 1 (40 μM) was added. For each experiment, cells treated with DMSO were used as control. After centrifugation, cells were lysed in PBS/0.32 M sucrose, pH 7.4, as described above. For the analysis of total cell proteome, the suspension was centrifuged for 20 min at 800 g at 4 °C, and the supernatant was saved (total lysate). For the experiments on cell fractions, the supernatant was centrifuged at 100000 g to obtain total cell membranes (pellet) and cytosol (supernatant). For each sample, extracted proteins were quantified using the BCA assay (Euroclone). The samples were stored at −80 °C until use. The click chemistry reaction was performed as described below.

Animals. We used male Sprague–Dawley rats, weighing 175–200 g (Charles River). All procedures were performed in accordance with the Ethical Guidelines of European Communities Council (Directive 2010/63/EU of 22 September 2010) and accepted by the Italian Ministry of Health. All efforts were made to minimize animal suffering and to use the minimal number of animals required to produce reliable results. Animals were group-housed in ventilated cages and had free access to food and water. They were maintained under a 12-h light/dark cycle (lights on at 8:00 am) at controlled temperature (21 ± 1 °C) and relative humidity (55% ± 10%). Experimenters were unaware of the treatment protocol at the time of the test (blind procedure).

Sample Preparation for CC-ABPP Analysis on Rat Lungs. Three rats per dose were used. Compound 1 or ARN726 was dissolved in PEG400/Tween 80/Saline solution at 10%/10%/80% in volume, respectively, and administered intravenously (iv) at a dose of 10 mg kg⁻¹. For the competition assay, rats were pretreated with ARN726 for 1 h, and then I was administered for additional 1 h. After treatments, the rats were sacrificed, and lungs were immediately dissected, frozen on dry ice, and stored at −80 °C until analyses. Lungs were homogenized in PBS, pH 7.4/0.32 M sucrose using an IKA T-18 Ultraturrax homogenizer. Samples were then centrifuged 25 min at 800g. The supernatant was centrifuged for 30 min at 12000g at 4 °C. The pellets were suspended in 2 volumes of PBS and subjected to two freeze/thaw cycles at −80 °C to solubilize proteins. The suspensions were centrifuged at 105000g for 1 h at 4 °C, and the soluble fraction was taken. For the in vitro experiments, lungs were obtained from naive rats (without treatment) and homogenized as described above. The supernatant obtained after the first centrifugation at 800g (total lysate) was centrifuged again either at 105000g to obtain total membranes (pellet fraction) and cytosol (soluble fraction) or at 120000 g as described above for the in vivo experiments. The membranes were suspended in 4 volumes of PBS. Protein concentration was measured by BCA assay (Euroclone), and samples were stored at −80 °C until use. The probe was added at 10 μM for 1 h at 37 °C. The click chemistry reaction was performed as described below.

Click Chemistry and Streptavidin Enrichment. The click chemistry and the streptavidin enrichment were performed according to the protocol described by Speers and Cravatt. Briefly, the following reagents were added to the protein preparations at the indicated final concentration: 100 μM azide–PEG3–biotin conjugate (CLK-AZ104P4, Jena Bioscience), 1 mM TCEP, 100 μM Tris(1-benzyl-1H-1,2,3-triazol-4-y1)methyl]amine (TBTA), 1 mM CuSO₄·5H₂O. TBTA was first dissolved in DMSO at 83.5 mM and then diluted with four volumes of t-butanol. The reaction was mixed by vortexing and incubated 1 h at 25 °C. The volumes of reaction were 2 × 500 μL (1 mg mL⁻¹ protein concentration) when streptavidin enrichment was required and 50 μL (1 mg mL⁻¹ protein concentration) when only protein blot analysis was performed. After the reaction time, the samples were either directly analyzed by protein blot or streptavidin enriched as described. Briefly, the proteins were first extracted by methanol/chloroform extraction; the final protein pellet was suspended in 650 μL of SDS 2.5% and sonicated 3 × 10 s, heated at 65 °C for 5 min, and sonicated again. Finally, the samples were centrifuged to remove the possible insoluble fraction. The percentage of SDS was diluted to 0.2% by PBS addition, and 100 μL of streptavidin–agarose resin (50% slurry, product no. 20353, Thermo Scientific) was added for 1 h at RT. The streptavidin beads were collected by centrifugation and washed with 1% SDS, 6 M urea, and PBS. The recovered beads were treated according to the further application.

ASSOCIATED CONTENT
S Supporting Information
Detailed methods, Supplementary Figures S1 and S2, and Supplementary Tables S1–S3. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00197.

AUTHOR INFORMATION
Corresponding Authors
*S.P. E-mail, stefano.ponzano@iit.it.
*D.P. E-mail, piomelli@uci.edu.

Author Contributions
*E.R. and S.P. contributed equally to this work.

Notes
The authors declare the following competing financial interest(s): Patent applications protecting the compounds disclosed in this paper were filed by the following authors: Daniele Piomelli, Stefano Ponzano, Fabio Bertozzi, and Tiziano Bandiera.

ACKNOWLEDGMENTS
We thank G. Ottonello, S. M. Bertozzi, L. Mengatto, and L. Goldoni for experimental help. We thank A. Fiasella and A. Nuzzi for kindly providing ARN726. This work was partially supported by the National Institute on Drug Abuse (to D.P.).

REFERENCES
(1) Tsboi, K., Sun, Y. X., Okamoto, Y., Araki, N., Tonai, T., and Ueda, N. (2005) Molecular characterization of N-acylethanolamine-
hydrolyzing acid amidase, a novel member of the choholylcine
hydrolyze family with structural and functional similarity to acid
ceramidase. J. Biol. Chem. 280, 11082–11092.
(2) Tsuboi, K., Takezaki, N., and Ueda, N. (2007) The N-
acylethanolamine-hydrolyzing acid amidase (NAAA). Chem. Biodiver-
sity 4, 1914–1925.
(3) Ueda, N., Tsuboi, K., and Uyama, T. (2010) N-acylethanolamine
metabolism with special reference to N-acylethanolamine-hydrolyzing
acid amidase (NAAA). Prog. Lipid Res. 49, 299–315.
(4) Rossocha, M., Schultz-Heienbrok, R., von Moeller, H., Coleman,
J. P., and Saenger, W. (2005) Conjugated bile acid hydrolyze is a
tetrameric N-terminal thiol hydrolyze with specific recognition of its
choyl but not of its tauryl product. Biochemistry 44, 5739–5748.
(5) Zhao, L. Y., Tsuboi, K., Okamoto, Y., Nagahata, S., and Ueda, N.
(2007) Proteolytic activation and glycosylation of N-acyl-
ethanolamine-hydrolyzing acid amidase, a lysosomal enzyme involved in
the endocannabinoid metabolism. Biochem. Biophys. Acta 1771, 1397–
1405.
(6) Wang, J., Zhao, L. Y., Uyama, T., Tsuboi, K., Tonai, T., and Ueda,
N. (2008) Amino acid residues crucial in pH regulation and
proteolytic activation of N-acylethanolamine-hydrolyzing acid amidase.
F. (2000) The endocannabinoid system as a target for
Acylethanolamine-hydrolyzing acid amidase (NAAA). Chem. Biodiver-
sity 4, 1914–1925.
(3) Ueda, N., Tsuboi, K., and Uyama, T. (2010) N-acylethanolamine
metabolism with special reference to N-acylethanolamine-hydrolyzing
acid amidase (NAAA). Prog. Lipid Res. 49, 299–315.
(4) Rossocha, M., Schultz-Heienbrok, R., von Moeller, H., Coleman,
J. P., and Saenger, W. (2005) Conjugated bile acid hydrolyse is a
tetrameric N-terminal thiol hydrolyse with specific recognition of its
choyl but not of its tauryl product. Biochemistry 44, 5739–5748.
(5) Zhao, L. Y., Tsuboi, K., Okamoto, Y., Nagahata, S., and Ueda, N.
(2007) Proteolytic activation and glycosylation of N-acyl-
ethanolamine-hydrolyzing acid amidase, a lysosomal enzyme involved in
the endocannabinoid metabolism. Biochem. Biophys. Acta 1771, 1397–
1405.
(6) Wang, J., Zhao, L. Y., Uyama, T., Tsuboi, K., Tonai, T., and Ueda,
N. (2008) Amino acid residues crucial in pH regulation and
proteolytic activation of N-acylethanolamine-hydrolyzing acid amidase.
F. (2000) The endocannabinoid system as a target for
Acylethanolamine-hydrolyzing acid amidase (NAAA). Chem. Biodiver-
sity 4, 1914–1925.
(3) Ueda, N., Tsuboi, K., and Uyama, T. (2010) N-acylethanolamine
metabolism with special reference to N-acylethanolamine-hydrolyzing
acid amidase (NAAA). Prog. Lipid Res. 49, 299–315.
(4) Rossocha, M., Schultz-Heienbrok, R., von Moeller, H., Coleman,
J. P., and Saenger, W. (2005) Conjugated bile acid hydrolyse is a
tetrameric N-terminal thiol hydrolyse with specific recognition of its
choyl but not of its tauryl product. Biochemistry 44, 5739–5748.
(5) Zhao, L. Y., Tsuboi, K., Okamoto, Y., Nagahata, S., and Ueda, N.
(2007) Proteolytic activation and glycosylation of N-acyl-
ethanolamine-hydrolyzing acid amidase, a lysosomal enzyme involved in
the endocannabinoid metabolism. Biochem. Biophys. Acta 1771, 1397–
1405.
amides as N-acylethanolamine-hydrolyzing acid amidase inhibitors. J. Med. Chem. 53, 5770−5781.

(35) Solorzano, C., Zhu, C., Battista, N., Astarita, G., Lodola, A., Rivara, S., Mor, M., Russo, R., Maccarrone, M., Antonietti, F., Duranti, A., Tontini, A., Cuzzocrea, S., Tarzia, G., and Piomelli, D. (2009) Selective N-acylethanolamine-hydrolyzing acid amidase inhibition reveals a key role for endogenous palmitoylethanolamide in inflammation. Proc. Natl. Acad. Sci. U.S.A. 106, 20966−20971.

(36) Vitale, R., Ottonello, G., Petracca, R., Bertozzi, S. M., Ponzano, S., Armirioti, A., Bertetti, A., Dionisi, M., Cavalli, A., Piomelli, D., Bandiera, T., and Bertozzi, F. (2014) Synthesis, structure-activity, and structure-stability relationships of 2-substituted-N-(4-oxo-3-oxetanyl) N-acylethanolamine acid amidase (NAAA) inhibitors. Angew. Chem., Int. Ed. 53, 8454−8458.

(37) Sasso, O., Moreno-Sanz, G., Martucci, C., Realini, N., Dionisi, M., Mengatto, L., Duranti, A., Tarozzo, G., Tarzia, G., Mor, M., Bertorelli, R., Reggiani, A., and Piomelli, D. (2013) Antinociceptive effects of the N-acylethanolamine acid amidase inhibitor ARN077 in rodent pain models. Pain 154, 350−360.

(38) Fiasella, A., Nuzzi, A., Summa, M., Armirioti, A., Tarozzo, G., Tarzia, G., Mor, M., Bertozzi, F., Bandiera, T., and Piomelli, D. (2014) 3-Aminoazetidin-2-one derivatives as N-acylethanolamine acid amidase (NAAA) inhibitors suitable for systemic administration. ChemMedChem 9, 1602−1614.

(39) Ribeiro, A., Pontis, S., Mengatto, L., Armirioti, A., Chiurchiu, V., Capurro, V., Fiasella, A., Nuzzi, A., Romeo, E., Moreno-Sanz, G., Maccarrone, M., Reggiani, A., Tarzia, G., Mor, M., Bertozzi, F., Bandiera, T., and Piomelli, D. (2015) A Potent Systemically Active N-Acylethanolamine Acid Amidase Inhibitor that Suppresses Inflammation and Human Macrophage Activation. ACS Chem. Biol., DOI: 10.1021/acschembio.5b00114.

(40) Armirioti, A., Romeo, E., Ponzano, S., Mengatto, L., Dionisi, M., Karacsonyi, C., Bertozzi, F., Garau, G., Tarozzo, G., Reggiani, A., Bandiera, T., Tarzia, G., Mor, M., and Piomelli, D. (2012) β-Lactones Inhibit N-acylethanolamine Acid Amidase by S-Acylation of the Catalytic N-Terminal Cysteine. ACS Med. Chem. Lett. 3, 422−426.

(41) Bottcher, T., andSieber, S. A. (2012) beta-Lactams and beta-lactones as activity-based probes in chemical biology. MedChemComm 3, 408−417.

(42) Staub, I., and Sieber, S. A. (2008) β-Lactams as selective chemical probes for the in vivo labeling of bacterial enzymes involved in cell wall biosynthesis, antibiotic resistance, and virulence. J. Am. Chem. Soc. 130, 13400−13409.

(43) Speers, A. E., and Cravatt, B. F. (2004) Profiling enzyme activities in vivo using click chemistry methods. Chem. Biol. 11, 535−546.

(44) Speers, A. E., and Cravatt, B. F. (2004) Chemical strategies for activity-based proteomics. ChemBioChem 5, 41−47.

(45) Kolb, H. C., Finn, M. G., and Sharpless, K. B. (2001) Click chemistry: Diverse chemical function from a few good reactions. Angew. Chem., Int. Ed. 40, 2004−2021.

(46) Lewis, W. G., Green, L. G., Grynszpan, F., Radic, Z., Carlier, P. R., Taylor, P., Finn, M. G., and Sharpless, K. B. (2002) Click chemistry in situ: Acetylcholinesterase as a reaction vessel for the selective assembly of a femtometer inhibitor from an array of building blocks. Angew. Chem., Int. Ed. 41, 1053−1057.

(47) Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) A stepwise Huisgen cycloaddition process: Copper(1)-catalyzed regioselective “ligation” of azides and terminal alkynes. Angew. Chem., Int. Ed. 41, 2596−2599.

(48) Wang, Q., Chan, T. R., Hilgraf, R., Fokin, V. V., Sharpless, K. B., and Finn, M. G. (2003) Bioconjugation by copper(1)-catalyzed azide-alkyne [3 + 2] cycloaddition. J. Am. Chem. Soc. 125, 3192−3193.

(49) Speers, A. E., and Cravatt, B. F. (2009) Activity-Based Protein Profiling (ABPP) and Click Chemistry (CC)-ABPP by MudPIT Mass Spectrometry. Curr. Protoc. Chem. Biol. 1, 29−41.

(50) Rawlings, N. D., Waller, M., Barrett, A. J., and Bateman, A. (2014) MEROPS: The database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res. 42, D503−D509.