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Authors

Armirotti, Andrea
Romeo, Elisa
Ponzano, Stefano
et al.

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β-Lactones Inhibit *N*-acylethanolamine Acid Amidase by S-Acylation of the Catalytic N-Terminal Cysteine

Andrea Armirotti,[†] Elisa Romeo,[†] Stefano Ponzano,[†] Luisa Mengatto,[†] Mauro Dionisi,[†] Claudia Karacsonyi,[†] Fabio Bertozzi,[†] Gianpiero Garau,[†] Glauco Tarozzo,[†] Angelo Reggiani,[†] Tiziano Bandiera,[†] Giorgio Tarzia,[∥] Marco Mor,[§] and Daniele Piomelli*,[†,‡]

[†]Department of Drug Discovery and Development, Istituto Italiano di Tecnologia, via Morego, 30, 16163 Genova, Italy
[‡]Departments of Pharmacology, University of California, Irvine, 360 MSRII, California 92697-4625, United States
[§]Pharmaceutical Department, University of Parma, 43124 Parma, Italy
[∥]Dipartimento di Scienze Biomolecolari, Università degli Studi di Urbino "Carlo Bo", Piazza del Rinascimento 6, I-61029 Urbino, Italy

S *Supporting Information*
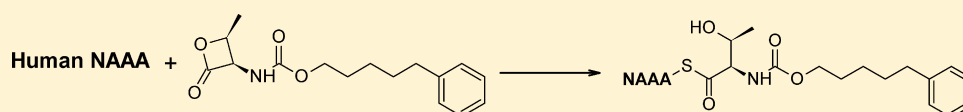
ABSTRACT: The cysteine amidase *N*-acylethanolamine acid amidase (NAAA) is a member of the N-terminal nucleophile class of enzymes and a potential target for anti-inflammatory drugs. We investigated the mechanism of inhibition of human NAAA by substituted β-lactones. We characterized pharmacologically a representative member of this class, **ARN077**, and showed, using high-resolution liquid chromatography−tandem mass spectrometry, that this compound forms a thioester bond with the N-terminal catalytic cysteine in human NAAA.

KEYWORDS: *NAAA, cysteine amidase, covalent inhibitors, high resolution mass spectrometry, proteomics*

The fatty acid ethanolamides (FAEs) are a family of bioactive lipid molecules that have attracted considerable interest due to their potential role in the control of pain, inflammation, and energy metabolism. Two structurally and functionally distinct classes of FAEs have been described. Polyunsaturated FAEs, such as arachidonoylethanolamide (anandamide) and eicosapentaenoylethanolamide, are endogenous agonists of G protein-coupled cannabinoid receptors.[1,2] These compounds are thought to act as retrograde messengers at synapses of the central nervous system and as local mediators in peripheral tissues. In the brain, anandamide-mediated signaling at CB_1 type cannabinoid receptors has been implicated in the regulation of anxiety,[3] depression,[4,5] stress-induced analgesia,[6] and nausea.[7] Outside the brain, anandamide released at sites of tissue injury is thought to control the initiation of emerging pain signals,[8,9] presumably by reducing the local release of proalgesic and proinflammatory factors from nociceptive nerve terminals.[10] The biological actions of anandamide are interrupted by a two-step deactivation process consisting of carrier-mediated transport into cells[11,12] followed by intracellular hydrolysis, which is catalyzed by the membrane-bound serine hydrolase, fatty acid amide hydrolase (FAAH). Inhibitors of this enzyme protect anandamide from deactivation and selectively magnify its intrinsic actions, producing an array of pharmacological effects that include reduced anxiety, depression, and pain.[13] Saturated and monounsaturated FAEs, which include oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), do not productively interact with cannabinoid receptors. These compounds are, however, potent or moderately potent

agonists of nuclear peroxisome proliferator-activated receptor-α (PPAR-α), which is responsible for most of their analgesic and anti-inflammatory properties.[14−16] OEA and PEA are produced in many mammalian tissues, including neurons[17] and innate immune cells,[18] where a selective phospholipase D releases them by cleaving the membrane precursor, *N*-acyl-phosphatidylethanolamine.[19] They are deactivated either by FAAH, which prefers anandamide and OEA over PEA, or by NAAA, which conversely prefers PEA and OEA over anandamide.[20] Despite its functional similarity with FAAH, NAAA shares no homology with this or other enzymes of the same "amidase signature" family. Rather, NAAA is an N-terminal nucleophile (Ntn) hydrolase and belongs to the choloylglycine hydrolase family of hydrolases, which are characterized by the ability to cleave nonpeptide amide bonds. Like other Ntn enzymes, NAAA is converted by proteolysis into a shorter active form upon incubation at acidic pH.[20] Considering its predominant localization in lysosomes,[21] it is conceivable that NAAA finds within these organelles the acidic pH (≈5.0) necessary for autocatalytic proteolysis and activation. Processing of NAAA renders a cysteine residue (Cys131 in mice and rats and Cys126 in humans) the N-terminal amino acid and catalytic nucleophile.[18,21,22] Pharmacological blockade of intracellular NAAA activity results in a normalization of OEA and PEA
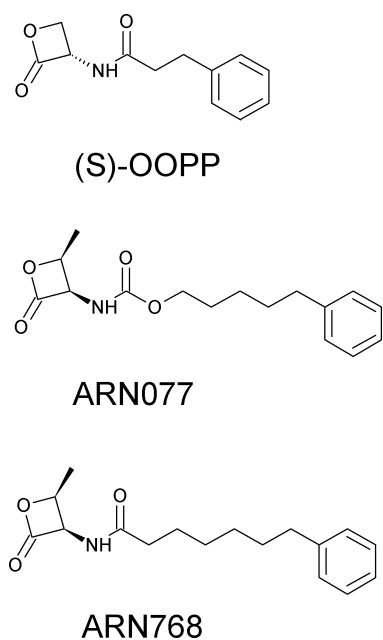
(S)-OOPP

ARN077

ARN768

Figure 1. Chemical structures of β-lactone NAAA inhibitors, (S)-N-(2-oxo-3-oxetanyl)-3-phenylpropanamide [(S)-OOPP], N-[(2S,3R)-2-methyl-4-oxo-oxetan-3-yl] carbamate (**ARN077**), and N-[(2S,3R)-2-methyl-4-oxo-oxetan-3-yl]-7-phenyl-heptanamide (ARN768).
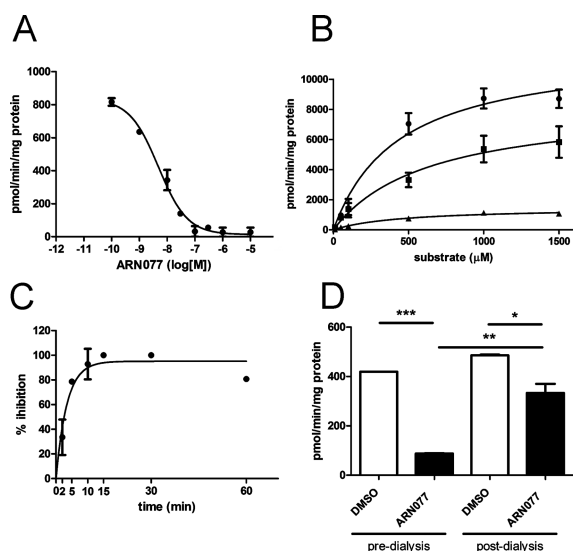

Figure 2. Inhibition of human NAAA by **ARN077**. (A) Concentration−response curve for inhibition of recombinant hNAAA by **ARN077** after 30 min of incubation. (B) Michaelis−Menten analysis of **ARN077** (vehicle, ●; 10 nM **ARN077**, ■; and 30 nM **ARN077**, ▲). (C) Preincbation time course of **ARN077** (100 nM). (D) Reversibility study of **ARN077** (vehicle, open bars; **ARN077**, black bars). *$p < 0.05$ **ARN077** postdialysis vs vehicle postdialysis; **$p < 0.01$ **ARN077** postdialysis vs **ARN077** predialysis; and ***$p < 0.001$ **ARN077** predialysis vs vehicle predialysis.

levels, which are markedly reduced in inflammatory states,[18,23,24] and exerts profound anti-inflammatory effects in animal models.[18] These findings suggest that NAAA inhibition might represent a novel mechanistic approach to control inflammation. A few groups of NAAA inhibitors have been developed to test this hypothesis.[18,25,26] Among them is a class
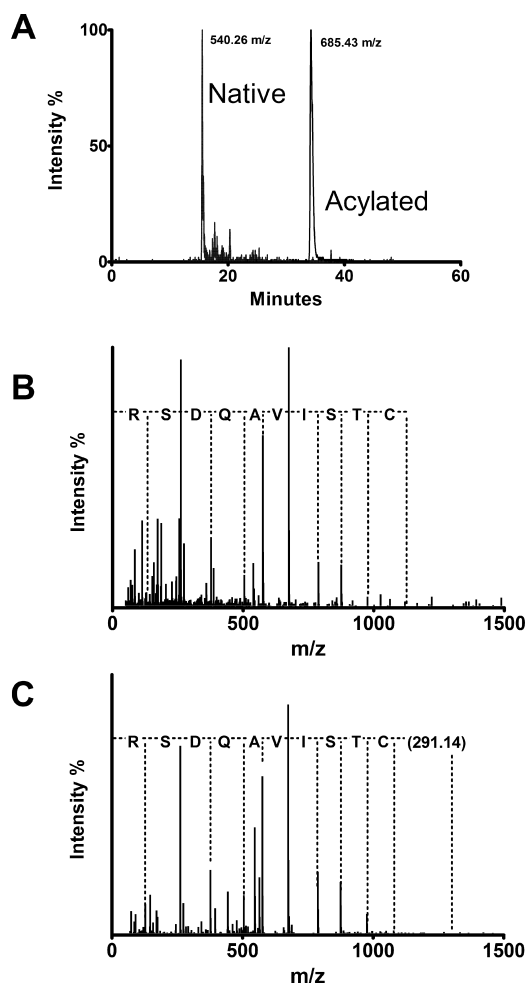

Figure 3. (A) Extracted ion chromatograms of the native (540.26 m/z) and acylated (685.43 m/z) peptide (for clarity purposes, peaks have not been normalized). (B and C) MS/MS spectra of m/z 540.26 and m/z 685.43. In both spectra, the y fragment ions series perfectly match a CTSIVAQDSR sequence (bearing a side chain adduct on the cysteine in the second case).

of substituted β-lactones, which shows promising potency and target selectivity.[18,26] The prototype of this class, (S)-N-(2-oxo-3-oxetanyl)-3-phenylpropanamide [(S)-OOPP, Figure 1], is a potent noncompetitive NAAA inhibitor that is highly effective at preventing PEA and OEA hydrolysis in activated inflammatory cells and at reducing tissue reactions to various pro-inflammatory stimuli.[18]

We have recently used the structure of (S)-OOPP as a starting point to discover β-lactone-based NAAA inhibitors with improved potency. This investigation lead us to identify several new compounds that selectively inhibit NAAA at low nanomolar concentrations, including 5-phenylpentyl N-[(2S,3R)-2-methyl-4-oxo-oxetan-3-yl] carbamate (**ARN077**, Figure 1). The structure−activity relationship properties of **ARN077** will be described elsewhere (Ponzano, S.; et al. Submitted for publication). In the present study, we used this molecule as a probe to characterize the mechanism through which substituted β-lactones inhibit NAAA. Our results indicate that the N-terminal cysteine of human recombinant NAAA (h-NAAA) is acylated by **ARN077** and that the new bond formed is a thioester between the cysteine thiol and the carbonyl group of the β-lactone.

ARN077 (Figure 1) inhibited the activity of h-NAAA with a median effective concentration (IC_{50}) of 7.3 ± 1.6 nM ($n = 3$) (Figure 2A) and a Hill coefficient of -0.8. By contrast, at the concentrations tested (up to 30 μM), the compound had no significant effect on the activity of FAAH or acid ceramidase, a cysteine amidase that exhibits 33−35% amino acid identity with NAAA[27] (Supporting Information, Table 1). Kinetic and time−course experiments showed that the inhibitory effect of **ARN077** was noncompetitive (Figure 2B and Supporting Information, Table 1) and reached half-maximal inhibition in 2.4 min and maximum inhibition within 10 min of incubation with the enzyme (Figure 2C).

Following centrifugation through a dialyzing membrane, the inhibition of h-NAAA activity produced by **ARN077** (100 nM) was markedly reduced but not completely reversed (Figure 2D). The results indicate that **ARN077** is a potent h-NAAA inhibitor that acts through a noncompetitive and partially reversible mechanism. Compounds that contain a β-lactone ring are known to interact covalently with biological nucleophiles, and some have been shown to inhibit Ntn enzymes such as NAAA.[26] This led us to hypothesize that **ARN077** might interact with h-NAAA through a covalent binding to the enzyme's catalytic cysteine. h-NAAA is translated as a 331 amino acid protein, which undergoes autoproteolytic cleavage at acidic pH 4.5 forming a new N-terminus, Cys126, which serves as the catalytic nucleophile.[28] We examined, therefore, whether **ARN077** might form a covalent adduct with the N-terminal cysteine of the active form of h-NAAA. If present, this modification should be carried by the corresponding N terminus peptide produced by trypsin digestion (C_{126}TSIVAQDS**R**; MW 1078.51). The position of this peptide within the primary sequence of the expressed h-NAAA is underlined below, along with the position of the trypsin cleavage sites (in bold):

M**R**TAD**R**EA**R**PGLPSLLLLLLAGAGLSAASP-PAAP**R**FNVSLDSVPEL**R**WLPVL**R**HYDLDLV**R**AA-MAQVIGD**R**VP**K**WVHVLIGKVVLELE**R**FLPQPFT-GEI**R**GMCDFMNLSLADCLLVNLAYESSVF<u>C</u>₁₂₆<u>TSIVA-QDSR</u>GHIYHG**R**NLDYPFGNVL**RK**LTVDVQFL**K**NG-QIAFTGTTFIGYVGLWTGQSPH**K**FTVSG-DE**R**D**K**GWWWENAIAALF**RR**HIPVSWLI**R**ATLSESEN-FEAAVG**K**LA**K**TPLIADVYYIVGGTSP**R**EGV-VIT**R**N**R**DGPADIWPLDPLNGAWF**R**VETNYDHW**K**-PAP**K**EDD**RR**TSAIKALNATGQANLSLEALF-QILSVVPVYNNLTIYTTVMSAGSPD**K**YM-T**R**I**R**NPS**RK**

A control incubation of h-NAAA at pH 4.5 for 90 min yielded a peptide of mass-to-charge ratio (m/z) 540.26 (Figure 3A), which was unambiguously identified as the product of auto-proteolytic cleavage of NAAA (Figure 3B), because the corresponding MS/MS spectrum shows a complete match of the y fragment ions series.[29] We next searched, in incubations of intact h-NAAA with **ARN077** (both at 5 μM), for adducts that might be generated by the nucleophilic attack of the thiol group of Cys126 on **ARN077**. As illustrated in Scheme 1, three such adducts are expected to be produced: the thioether a, formed through S-alkylation of the carbon in the 2-position of the oxooxetan ring of **ARN077**; the thioester b, formed through S-acylation of the oxooxetan carbonyl carbon of **ARN077**; and the thioesters c and c′, formed by the attack of Cys 126 on the carbamate group of **ARN077**.

Scheme 1. Possible Nucleophylic Attacks of Cysteine on ARN077[a]
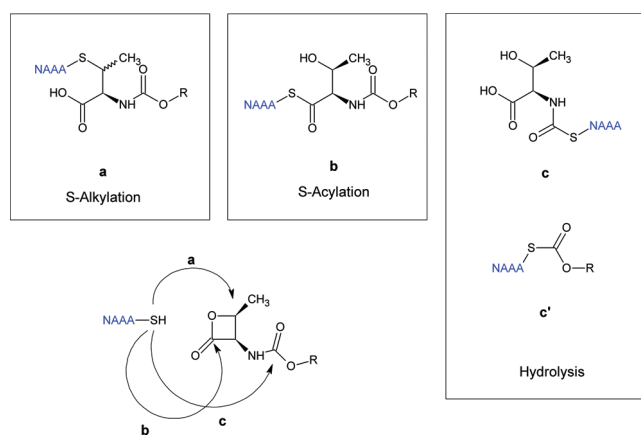
[a] Path a, thioether adduct; path b, thioester adduct; and path c, hydrolysis of the carbamate, with two possible adducts (c and c′). R = $(CH_2)_5$-Ph.

The adducts a and b are isomers of formula $C_{58}H_{95}N_{15}O_{21}S$ and are expected each to generate a doubly charged ion of m/z 685.43. A peptide with this m/z value was eluted approximately 20 min after the native, unmodified peptide (Figure 3A). MS/MS analysis of this component (Figure 3C) produced a y fragment ion series that matched that of the native peptide (Figure 3B), clearly indicating that **ARN077** covalently links to the N-terminal cysteine of NAAA. The presence in the MS/MS spectrum of an ion of m/z 292.16, together with the corresponding loss of water (m/z 274.14), indicate a fragmentation of the acylated cysteine side chain ($C_{16}H_{22}NO_4$). This is confirmed by the presence of the singly charged peptide ion at m/z 1079.52. However, this fragmentation did not allow us to conclusively determine whether the broken bond was a thioester or a thioether. Diagnostic ions that would permit to discriminate between S-alkylation and S-acylation are expected to be formed upon C−C cleavage of the thioether or α-cleavage of the thioester (Supporting Information, Figure 1). The former, which should produce an ion of m/z 264.12 ($C_{14}H_{18}NO_4$), was not detected in our analyses, while the latter, which should produce an ion of m/z 264.16 ($C_{16}H_{24}NO_2$), is rather weak and was covered by the second isotopic peak (m/z 264.16) of the very intense y_2 ion of the backbone fragmentation at m/z 262.15. These results tentatively suggest that **ARN077** might form a thioester bond with the N-terminal cysteine of h-NAAA, but additional evidence is needed to draw such conclusion. To fill this gap, we tested the ability of the compound **ARN768** (Figure 1), an amide analogue of **ARN077** that is a relatively potent h-NAAA inhibitor (IC_{50} 314 $\pm$ 52 nM ; $n = 3$), to interact covalently with h-NAAA. We found that ARN0768 reacts with h-NAAA forming, as does **ARN077**, a peptide adduct whose MS/MS spectrum shows a side chain fragment ion at m/z 290.18 ($C_{17}H_{24}NO_3$), the corresponding loss of water at m/z 272.17, and a clearly detectable ion at m/z 262.18 (Figure 4).

The latter corresponds to the cleavage of a thioester ($C_{15}H_{22}NO_3$) rather than a thioether ($C_{15}H_{20}NO_3$), whose corresponding ion at m/z 262.14 was not detected. Notably, attack on the carbamate group (mechanism c in Scheme 1) is expected to produce the modified peptides c ($C_{47}H_{81}N_{15}O_{21}S$) and c′ ($C_{54}H_{88}N_{14}O_{19}S$), with the alkyl-phenyl chain or the
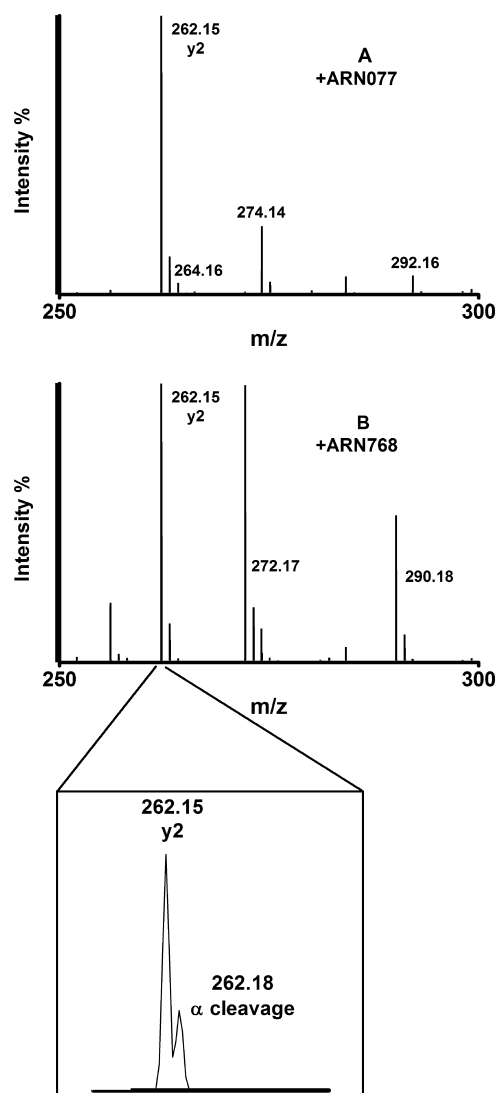
Figure 4. Zoomed MS/MS spectra of peptide CTSIVAQDSR acylated by **ARN077** and **ARN768**. Cysteine side chain fragment ions are indicated along with the y2 fragment (262.15 m/z). S-Acylation diagnostic fragment ion at 262.18 m/z is reported in the inset.

essential tool for the direct validation of reaction mechanisms that are important in drug discovery. In the present case, the high-resolution tandem mass capabilities of the hybrid quadrupole-TOF instrument allowed us to unequivocally assign the structure of side chain fragment ions, which resulted in an unambiguous assignment of the chemical structure of the modified peptide. Furthermore, our results provide a solid mechanistic basis for the identification of a second generation of β-lactone-based NAAA inhibitors with improved potency, selectivity, and metabolic stability.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

Detailed description of the experimental procedures, MS/MS fragmentation analysis, and pharmacological data of **ARN077**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Prof. Daniele Piomelli Department of Drug Discovery and Development, Istituto Italiano di Tecnologia, via Morego, 30, 16163 Genova. Email: Daniele.piomelli@iit.it.

Author Contributions

AA, GG, FB designed the experiments and analyzed the data. SP synthesized β-lactones. GT, MD and LM performed pharmacological profiling of **ARN077**. ER and CK produced the recombinant h-NAAA. DP oversaw the project with assistance from TB, AR, GT, MM. AA and DP wrote the manuscript.

Notes

The authors declare the following competing financial interest(s): A patent protecting **ARN077** and related compounds was filed by the following authors: Piomelli, D.; Bandiera, T.; Mor, M.; Tarzia, G.; Bertozzi, F.; Ponzano, S.

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threonine residue as respective leaving groups. These peptides were not detected in our experiments, and the corresponding mechanism was therefore tentatively excluded. Furthermore, no evidence of adduct formation was found when the *h*-NAAA holoenzyme was incubated with **ARN077**, indicating that only the active form of the enzyme is subjected to S-acylation by this compound. Together, these findings suggest that substituted β-lactones, such as **ARN077** and **ARN768**, react with Cys126 in *h*-NAAA to form a thioester bond. Moreover, we found no evidence of other peptide adducts with either inhibitor, suggesting that β-lactones selectively interact with the *N*-terminus cysteine.

The high-resolution mass spectrometry studies reported here indicate that the substituted β-lactones, **ARN077** and **ARN768**, inhibit *h*-NAAA activity through a mechanism that requires S-acylation of the catalytically active N-terminal cysteine residue of this enzyme. These findings are consistent with the pharmacological properties of **ARN077**, which blocks *h*-NAAA activity in a noncompetitive and partially reversible manner (as expected from the covalent yet hydrolyzable nature of a thioester bond). The results underscore the value of bottom-up proteomics as an

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