Experimental Procedures

Vector construction and cell transfection

The coding sequence of *h*-NAAA (NM_014435.3) was first amplified by PCR (HotStar HiFidelity kit, Qiagen) from a human spleen cDNA library (cat# 639124 Clontech, CA). Then to generate the construct *h*-NAAA(6xHis)-pCDNA3.1 a sequence of *h*-NAAA fused with a C-terminal 6xHis tag was amplified [forward primer: 5'-AAGCTTGAGCCCGAGCCATGCGGACCG-HindIII restriction site is underlined; reverse primer: 5'-CTCGAGTTA**GTGGTGGTGGTGGTGGTG**CTTTCTACTCGGGTTTC-XhoI restriction sites is underlined, the histidine tag is in bold] and cloned into HindIII/XhoI digested pCDNA 3.1 vector (Invitrogen,CA). HEK293 wild-type cells (American Type Culture Collection, Manassas, VA) were cultured at 37°C in a humidified incubator (5% CO_2) using Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin-streptomycin, and transfected with the *h*-NAAA(6xHis)-pCDNA3.1 construct using the JetPEI transfection reagent (Polyplus, Illkirch FR), following manufacturer's instructions. Stably transfected cells were selected by addition of G418 1mg/ml to the cell culture media and cell clones were generated by limiting dilution plating. Growing clones were analyzed by western blot (mAb anti-hASAHL, R&D Systems) for their capacity to express *h*-NAAA.

***h*-NAAA expression and purification.**

The best producing *h*-NAAA-HEK clone was adapted to serum-free growing condition (Free style medium, Invitrogen, CA) and cultivated in spinning culture at 37°C in a humidified incubator (8% CO_2). The cell medium was harvested every 48 h and stored at -20°C until used. For purification, 400 ml of clarified cell supernatant were incubated with 3 ml of NiNTA (Qiagen) overnight at 4°C with stirring. The collected affinity resin was washed with a buffer of 20 mM Hepes pH 7, 300 mM NaCl, 20 mM imidazole. Proteins were eluted with the same buffer containing 500 mM imidazole and then dialyzed overnight with a buffer without imidazole.

Synthesis of 5-phenylpentyl-*N*-[(2S,3R)-2-methyl-4-oxooxetan-3-yl]-carbamate (ARN077)

- Preparation of 5-phenylpentyl-chlorocarbonate

In a four necked round bottom flask, triphosgene (22.0 g, 74.2 mmol) was dissolved in toluene (325 ml) under argon. After cooling at 0 °C, pyridine (19.2 ml, 237.5 mmol) was added dropwise during 1 h and the resulting suspension stirred at 0 °C for 1 h. 5-phenylpentanol (25 ml, 148.4 mmol) was added dropwise for a period of 30 min. After stirring at room temperature for 16 h the solid was filtered-off and the solvent removed under vacuum yielding the title compound (31.7 g, 94%), which was used in the next step without further purification. R_f = 0.75 (Cy/AcOEt 8:2). FTIR (cm^{-1}): 3087, 3063, 3027, 2936, 2859, 1778, 1744, 1604, 1496, 1455, 1382, 1260, 1146, 1031, 941, 835, 731, 696.

- Preparation of (2*R*,3*S*)-3-hydroxy-2-({[(5-phenylpentyl)-oxy]-carbonyl}-amino)-butanoic acid

In a round bottom flask, $NaHCO_3$ (12.0 g, 143.2 mmol) was suspended in THF (30 ml) and H_2O (60 ml), then *D*-threonine (6.8 g, 56.8 mmol) was added followed by 5-phenylpentyl chlorocarbonate (14.3 g, 63.1 mmol) and a catalytic amount of tetrabutylamonium bromide (0.67 g). After stirring at room temperature for 18 h, the mixture was diluted with water, washed twice with Et_2O and pH adjusted to 2 with 2M HCl solution. The aqueous phase was extracted with AcOEt (4x150 ml), the collected organic phases were washed with brine, dried over Na_2SO_4, filtered and the solvent removed under vacuum. The crude was purified by typical silica gel column chromatography, eluting with Cy/AcOEt (20:80 +1% CH_3COOH), yielding the title compound (3.15 g, 18%), as pale yellow oil. R_f = 0.38 (Cy/AcOEt 2:8 + 1% CH_3COOH). FTIR (cm^{-1}): 3332 (br), 3027, 2935, 2859, 1714, 1530, 1454, 1415, 1257, 1072, 1007, 963, 870, 779, 748, 700. ^1H NMR ($CDCl_3$): □ 1,26 (d, 3H); 1,39 (m, 2H); 1,62-1,68 (m, 4H); 2.61 (t, 2H); 4.08 (t, 2H); 4.34 (d, 1H); 4.42 (d, 1H); 5.68 (d, 1H); 7.16-7.78 (m, 3H); 7.26-7.28 (m, 2H) ppm.

- Preparation of 5-phenylpentyl-*N*-[(2*S*,3*R*)-2-methyl-4-oxooxetan-3-yl]-carbamate (**ARN077**)

In a two necked round bottom flask, Et_3N (0.34 ml, 2.42 mmol) was added to a solution of (2R,3S)-3-hydroxy-2-({[(5-phenylpentyl)-oxy]-carbonyl}-amino)-butanoic acid (0.25 g, 0.81 mmol) in dry CH_2Cl_2 (10 ml) under argon. After cooling at 0 °C, O-benzotriazole-N,N,N',N'-tetramethyl-uronium hexafluorophosphate (HBTU) (0.46 g, 1.21 mmol) was added and the mixture stirred at 0 °C for 3 h, then at room temperature for 16 h. The obtained solid was filtered-off and the solvent removed under vacuum. The crude was purified by typical silica gel column chromatography eluting with Cy/AcOEt (from 98:2 to 80:20). The title compound was obtained (0.107 g, 45%) as white solid, which was further triturated with cyclohexane. MS (ESI) m/z: 292 [M-H]+; (ESI) m/z: 290 [M-H]-. FTIR (cm-1): 3330, 3064, 3027, 2936, 2858, 1849, 1695, 1540, 1387, 1333, 1262, 1129, 1076, 1023, 985, 921, 845, 822, 741, 697. 1H NMR (DMSO-d6): δ 1.33 (d, J = 6.4 Hz, 3H), 1.28–1.38 (m, 2H), 1.53–164 (m, 4H), 2.56 (t, J = 7.7 Hz, 2H,), 3.94–4.05 (m, 2H), 4.84, (dq, J = 6.2, 6.4 Hz), 5.41 (dd, J = 6.2, 9.4 Hz, 1H), 7.13–7.31 (m, 5H), 8.20 (d, J = 9.4 Hz, 1H) ppm. 13C NMR (DMSO-d6): δ 14.5, 25.0, 28.3, 30.6, 35.1, 59.9, 64.7, 74.7, 125.7, 128.2, 128.3, 142.1, 155.8, 169.9 ppm.

Synthesis of N-[(2S,3R)-2-methyl-4-oxo-oxetan-3-yl]-7-phenyl-heptanamide (ARN768).

- Preparation of (2*S*,3*R*)-2-methyl-4-oxo-3-oxetanylammonium toluene-4-sulfonate**.**

White solid; experimental procedure and ^{1}H NMR are according to literature. (ref [1] and refs therein).

- Preparation of *N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]-7-phenyl-heptanamide (**ARN768**).

To a stirred mixture of 7-phenylheptanoic acid (0.083 g, 0.40 mmol) in dry CH_2Cl_2 (3.0 ml) under nitrogen atmosphere, O-(benzotriazol-1-yl)-*N,N,N′,N′*-tetra methyluronium tetra fluoroborate (TBTU) (0.129 g, 0.40 mmol) and Et_3N (0.05 ml, 0.40 mmol) were added. The reaction mixture was left stirring at room temperature for 15 min. Subsequently a solution of (2*S,*3*R*)-2-methyl-4-oxo-3-oxetanylammonium toluene-4-sulfonate (0.10 g, 0.36 mmol) and Et_3N (53 ml, 0.40 mmol) in CH_2Cl_2 (2.0 ml) was added. The reaction mixture was left stirring at room temperature for 16 h. The solvent was rotary evaporated and the crude product was purified by column chromatography, eluting with Cyclohexane/EtOAc (from 100:0 to 30:70) to afford the title compound (0.045 g, 42 %), as a white solid. MS (ESI) *m/z*: 290 $[M-H]^+$; (ESI) *m/z*: 288 $[M-H]^-$. ^{1}H NMR (DMSO-d_6): δ 1.24–1.34 (m, 4H), 1.31 (d, *J* = 6.4 Hz, 3H), 1.45–1.61 (m, 4H), 2.16 (t, *J* = 7.3 Hz, 2H), 2.52–2.60 (m, 2H), 4.84 (dq, *J* = 6.0, 6.4 Hz, 1H), 5.54 (dd, *J* = 6.0, 8.7 Hz, 1H), 7.12–7.29 (m, 5H), 8.74 (d, *J* = 8.7 Hz, 1H) ppm. ^{13}C NMR (DMSO-d_6): δ 14.63, 24.98, 28.30, 30.85, 34.86, 35.06, 58.11, 74.41, 125.56, 128.18, 128.20, 142.23, 169.96, 172.47 ppm.

Sample Preparation for Proteomics

Purified *h*-NAAA (5μM) was incubated in 100 mM Sodium Phosphate Buffer, 100 mM Sodium Citrate, 3 mM Tris(2-carboxyethyl)phosphine (TCEP), 0.1% Triton X100, pH 4.5 for 90 min at 37°C. ARN077 was added to a final 5μM concentration and the solution was incubated for 90 min at 37°C (final DMS0 concentration, 0.5%). A control sample incubated with 0.5% DMSO was also prepared. At the end of the incubation, samples were precipitated with 10 volumes of ice cold acetone, vortexed and centrifuged at 14,000 rpm for 10 min. The supernatants were removed and *h*-NAAA was suspended with Rapigest 0.5% (Waters, USA) and digested with immobilyzed trypsin [2] (DigestTip Trypsin, ProteoGen Bio, Italy). Prior to digestion, *h*-NAAA autocleavage was verified by SDS-PAGE.

High Resolution NanoLC-MS/MS Analysis

Tryptic peptides were loaded in a NanoAcquity LC system coupled with a Synapt G2 qTOF mass spectrometer (Waters, USA) equipped with a NanoSpray ion source. The analytical column was a Waters BEH C18 75 μm x 10 cm. Flow rate was set to 300 nl/min. Eluents were: A=water plus 0,1% formic acid, B=acetonitrile plus 0,1% formic acid. A linear gradient from 3% to 55%B in 30 min was applied to the column followed by a ramp to 90% in 10 minutes, an isocratic step at 90% for 10 minutes and a reconditioning to 3% B. Mass spectrometry parameters were: spray 1.8KV, cone 25V, source temp 150°C, cone flow 50 l/min. Data Dependent Acquisition of tandem mass spectra was activated for doubly charged ions in the *m/z* 300-800 range. A linear ramp of the collision energy from 15 to 35eV was applied to the precursor ion to collect tandem mass spectra. 500 nM glucofibrinopeptide infused at 500 nl/min was used as

lockspray mass. MS/MS data were analyzed using the Biolynx software embedded in the MassLynx software suite. MassLynx and ProteinLynx softwares (Waters, USA) were used for the interpretation of LC-MS data.

NAAA activity

Recombinant *h*-NAAA-expressing HEK293 cells were washed in PBS and scraped from flasks, collected in 50ml Falcon tubes on ice and centrifuged at 450×g for 10 min at 4°C. Cell pellets were suspended in 20 mM Tris-HCl buffer pH 7.4, 320 mM sucrose, and sonicated. Samples were then centrifuged at 800×g for 15 min at 4°C and the resultant supernatants were ultracentrifuged at 12,000×g for 30 min at 4°C. The pellets were suspended in PBS (100 mM, pH 7.4) on ice and subjected to a freeze/thaw cycle at -80°C. The suspensions were centrifuged at 105,000×g for 1 h at 4°C. Protein concentration was measured and samples were divided into aliquots and stored at -80°C until use. *h*-NAAA protein (10 μg) was incubated with ARN077 or vehicle in 100 mM NaH_2PO_4, 100 mM Tri Sodium Citrate Dehydrate, 0.1% Triton-X 100, 3 mM dithiothreitol, pH 4.5 for 30 min at 37°C. Duplicate samples were incubated with 50 μM 10-cis-heptadecenoylethanolamide (Avanti Polar Lipids, Alabaster, AL-USA) at 37°C for 30 min. The reaction was terminated by the addition of cold methanol (0.2 mL) spiked with 5μM heptadecanoic acid (NuChek Prep, Elysian, MN-USA) as internal standard. Samples were analyzed by UPLC/MS using a single quadrupole detector (Waters, USA) operating in the negative ionization mode. Heptadecenoic and heptadecanoic acids were separated on an Acquity UPLC BEH C18 column (50 mm length, 2.1 mm i.d., 1.7 μm pore size, Waters, USA) with an isocratic run at 0.5 ml/min for 1.5 min in isocratic mode, using 95% methanol and 5% water, both containing 0.25% acetic acid and 5 mM ammonium acetate. The column temperature was set to 40 °C. Capillary voltage was set to 2.7 kV and cone voltage to 45 V. The source temperature was 150°C with a desolvation temperature of 400°C. N_2 was used as drying gas at a cone flow of 100 l/hour and a gas flow of 800 l/Hour. The [M-H]- ions were monitored in selected-ion monitoring mode (m/z values: heptadecenoic acid = *m/z* 267; heptadecanoic acid = *m/z* 269). Calibration curves were generated using commercial heptadecenoic acid (NuCheck Prep). IC_{50} values were calculated by non-linear regression analysis of log[concentration]/inhibition curves using GraphPad Prism 5 (GraphPad Software Inc., CA–USA) applying a standard slope curve fitting.

Kinetic and reversibility analyses

Assay conditions for the kinetic studies were the same as those described for standard NAAA assays. Substrate (10-cis-heptadecenoylethanolamide, Avanti Polar Lipids) was added at the following concentrations (μM): 10, 50, 100, 500, 1000, 1500. ARN077 was used at a final concentration of 10 nM or 30 nM. For reversibility studies, *h*-NAAA (150 μg) and ARN077 (100 nM) or vehicle were incubated in NAAA assay reaction buffer (1.5 ml) for 30 min. A 0.5 ml sample was taken for immediate NAAA assay, while a 1 ml sample was diluted in 9 ml of assay buffer without Triton and centrifuged in Amicon Ultra-15 10K MWCO centrifugal filter devices (UFC901008, Millipore MA-USA) for 15 min at 1000×g. The latter step was repeated three times, substrate was added, and the sample was assayed for NAAA activity.

FAAH activity

ARN077 was incubated for 10 min in assay buffer (50 mM TRIS pH 7.4, 0.05% fatty acid-free bovine serum albumin) with rat brain homogenate (50 μg protein/tube). Substrate (1 μM non-radioactive anandamide and 0.6 nM (0.04 μCi/mL) [^{3}H]-anandamide (arachidonyl-[$^{1\text{-}3}$H] ethanolamine, specific activity 1.7 Ci/mmol; concentration 0.15 mCi/ml, NIDA Drug Supply Program; Bethesda, MD-USA) were incubated at 37°C for 30 min and reactions were stopped with cold $CHCl_3$/methanol (1:1). Radioactivity in the aqueous phase was measured by liquid scintillation counting (Microbeta2 Lumijet, Perkin Elmer Inc., MA-USA).

Acid ceramidase activity

ARN077 was incubated for 30 min at 37°C with 25 μg of enzyme derived from human acid ceramidase-overexpressing HEK293 cells, and *N*-lauroyl ceramide 100 μM (Nu-Chek Prep) was added as substrate, as described by Solorzano [1] with the following variations. Samples were analyzed using the same UPLC/MS system described above, working in

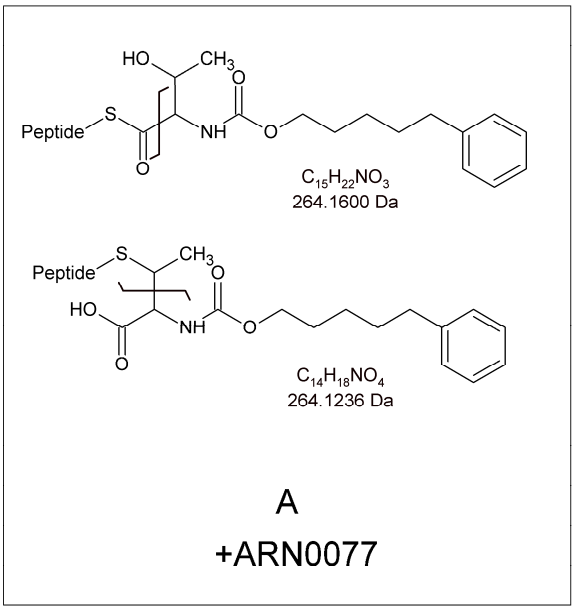
negative ion mode, and using dodecenoic acid (NuChek Prep) as internal standard (*m/z* = 199 for lauric acid, *m/z* = 197 for dodecenoic acid). Dodecanoic and dodecenoic acids were separated at 0.5 ml/min on an Acquity UPLC BEH C18 column (50 mm length, 2.1 mm i.d., 1.7 µm pore size) maintained at 40°C. Mobile phases consisted of A (water) and B (acetonitrile) both containing 0.25% acetic acid and 5 mM ammonium acetate. Separations were carried out using a gradient from 70% B to 100% in 0.5 minutes. Capillary voltage was set at 2.1 kV and cone voltage at 45 V. The source temperature was 150°C with a desolvation temperature of 400°C. N_2 was used as drying gas at a cone flow of 100 l/hour and a desolvation flow of 800°C. The $[M-H]^-$ ion was monitored in the selected-ion mode. Calibration curves were generated using commercial lauric acid (NuChek Prep).

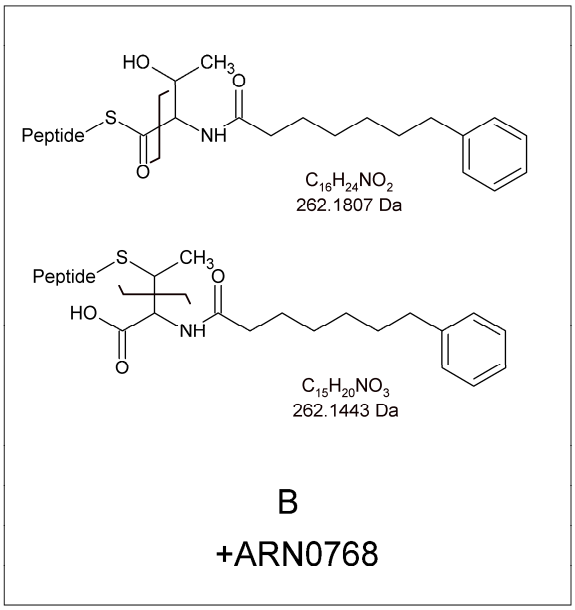
References

(1) Solorzano, C.; Antonietti, F.; Duranti, A.; Tontini, A.; Rivara, S.; Lodola, A.; Vacondio, F.; Tarzia, G.; Piomelli, D.; Mor, M. Synthesis and structure-activity relationships of N-(2-oxo-3-oxetanyl)amides as N-acylethanolamine-hydrolyzing acid amidase inhibitors,*J Med Chem* **2010**, *53*, 5770-5781.

(2) Marangoni, R.; Chiarini, R.; Iannone, G.; Salerno, M. DigesTip: a new device for a rapid and efficient in-solution protein digestion,*Proteomics* **2008**, *8*, 2165-2167.

Supporting Figure 1





Useful fragmentations for discriminating between S-alkylation and S-Acylation of ARN077 and ARN768

Supporting Tables 1 and 2 - Pharmacological data on ARN0077

Table 1

Enzyme	**IC50 ± SD (nM)**
human NAAA (recombinant)	7.3±1.6
Acid Ceramidase (human recombinant)	>100,000
FAAH (human recombinant)	>100,000

Table 2

		DMSO	**ARN0077 10nM**	**ARN0077 30nM**
hNAAA	Vmax (pmol/min/mg)	12,000	8,390	1,489
	Km (μM)	439	638	461