N-Cyclohexanecarbonylpentadecylamine: a selective inhibitor of the acid amidase hydrolysing *N*-acylethanolamines, as a tool to distinguish acid amidase from fatty acid amide hydrolase

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Anandamide (*N*-arachidonoylethanolamine) and other bioactive *N*-acylethanolamines are degraded to their corresponding fatty acids and ethanolamine. This hydrolysis is mostly attributed to catalysis by FAAH (fatty acid amide hydrolase), which exhibits an alkaline pH optimum. In addition, we have identified another amidase which catalyses the same reaction exclusively at acidic pH values [Ueda, Yamanaka and Yamamoto (2001) J. Biol. Chem. **276**, 35552–35557]. In attempts to find selective inhibitors of this acid amidase, we screened various derivatives of palmitic acid, 1-hexadecanol, and 1-pentadecylamine with *N*-palmitoylethanolamine as substrate. Here we show that *N*-cyclohexanecarbonylpentadecylamine inhibits the acid amidase from rat lung with an IC_{50} of 4.5 μM, without inhibiting FAAH at concentrations up to 100 μM. The inhibition was reversible and non-competitive. This compound also inhibited the acid amidase in intact alveolar macrophages. With the aid of this inhibitor, it was revealed that rat basophilic leukaemia cells possess the acid amidase as well as FAAH. Thus the inhibitor may be a useful tool to distinguish the acid amidase from FAAH in various tissues and cells and to elucidate the physiological role of the enzyme.

Key words: acid amidase, anandamide, endocannabinoid, macrophage, *N*-acylethanolamine, *N*-palmitoylethanolamine.

INTRODUCTION

Ethanolamides of long-chain fatty acids (collectively referred to as *N*-acylethanolamines) exist ubiquitously in animal tissues [1–3]. This class of lipids includes several bioactive compounds. *N*-Arachidonoylethanolamine (anandamide) is well known to be an agonist of cannabinoid receptors (CB1 and CB2) and the vanilloid receptor (VR1) [4,5]. *N*-Palmitoylethanolamine does not activate cannabinoid receptors, but shows anti-inflammatory and analgesic effects [6,7]. Furthermore, *N*-oleoylethanolamine has been reported to have an anorexic effect [8] through activation of the nuclear receptor PPAR-α (peroxisome proliferator-activated receptor-α) [9], and *N*-stearoylethanolamine has an apoptosis-inducing effect [10]. These *N*-acylethanolamines are hydrolysed to their corresponding fatty acids and ethanolamine by catalysis by FAAH (fatty acid amide hydrolase), a membrane-bound serine hydrolase [11–15]. Recent studies revealed high levels of anandamide and other *N*-acylethanolamines in the brains of FAAH gene-disrupted mice, confirming the central role of this enzyme in the degradation of *N*-acylethanolamines in brain [16–18].

However, when we screened various animal tissues and cells for *N*-acylethanolamine-hydrolysing activity at pH 5, we discovered another *N*-acylethanolamine hydrolase which acted exclusively at acidic pH in human megakaryoblastic CMK cells [19], and later in various rat tissues, with the highest specific activity in lung [20]. This 'acid amidase' was characterized by an optimal pH of 5, solubilization from the membrane by freezing and thawing, activation by Triton X-100 and DTT (dithiothreitol), and lower sensitivity to serine hydrolase inhibitors such as PMSF and MAFP (methyl arachidonoyl fluorophosphonate) [19,20]. Thus the acid amidase was catalytically distinguishable from FAAH. Although the enzyme could hydrolyse various *N*-acylethanolamines with different fatty acyl species, it was noted that *N*-palmitoylethanolamine was the most reactive substrate [20]. Therefore the enzyme will be referred to as NPAA, for *N*-palmitoylethanolamine-hydrolysing acid amidase, in this paper.

To distinguish NPAA from FAAH in *in vitro* and *in vivo* assays and to clarify the physiological roles of NPAA, selective enzyme inhibitors should be useful tools. While a vast number of FAAH inhibitors have been developed [11–14,21], selective inhibitors for NPAA have not yet been reported. Very recently, we found esters, retroesters and a retroamide of palmitic acid to be NPAA inhibitors, with a lower inhibitory effect on FAAH [22]. Cyclohexyl hexadecanoate (compound **1** in Figure 1) was the most potent among the compounds tested, but its IC_{50} was still higher than 10 μM. In order to develop more potent NPAA inhibitors, we further tested a series of compounds structurally related to compound **1**. In the present study, we report for the first time considerably potent NPAA inhibitors with IC_{50} values of < 10 μM, and describe an inhibitor that is useful for distinguishing between NPAA and FAAH.

MATERIALS AND METHODS

Materials

[1-^{14}C]Palmitic acid was purchased from Du Pont NEN (Boston, MA, U.S.A.), *N*-palmitoylethanolamine and MAFP from Cayman Chemical Company (Ann Arbor, MI, U.S.A.), DTT from Wako Pure Chemical (Osaka, Japan), Triton X-100 from Nacalai Tesque (Kyoto, Japan), precoated silica gel 60 F_{254} aluminum sheets for TLC (20 cm × 20 cm; 0.2 mm thickness) from Merck (Darmstadt, Germany), protein assay dye reagent concentrate from Bio-Rad (Hercules, CA, U.S.A.) and RPMI 1640 medium from Invitrogen (Carlsbad, CA, U.S.A.). *N*-[^{14}C]Palmitoylethanolamine was chemically synthesized from [1-^{14}C]palmitic

Abbreviations used: DTT, dithiothreitol; FAAH, fatty acid amide hydrolase; MAFP, methyl arachidonoyl fluorophosphonate; NPAA, *N*-palmitoylethanolamine-hydrolysing acid amidase; RBL, rat basophilic leukaemia.

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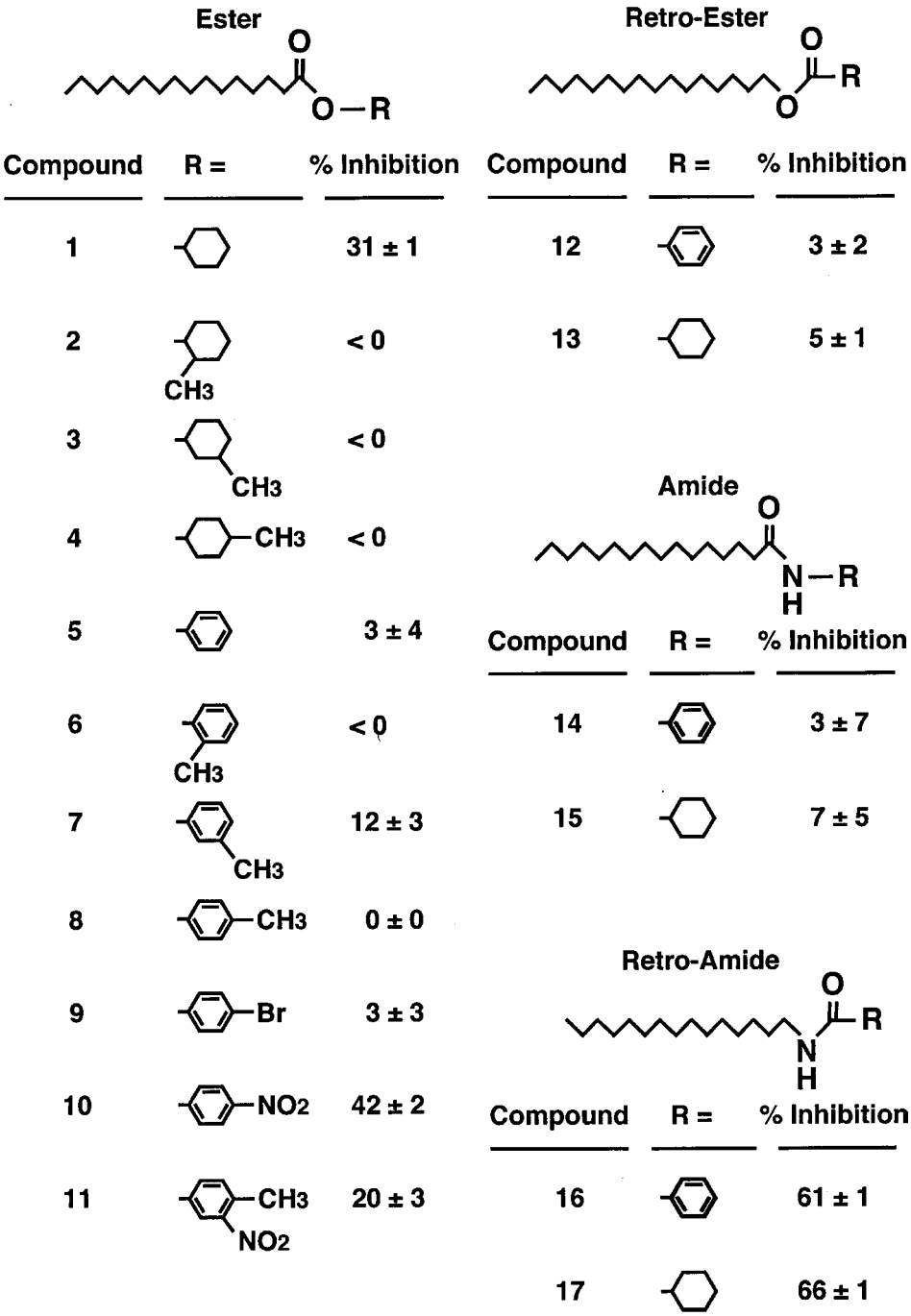

Figure 1 Inhibitory effects on NPAA of esters, retroesters, amides and retroamides of palmitic acid

Rat lung NPAA (11.7 μg of protein) was allowed to react with 100 μM *N*-[^{14}C]palmitoylethanolamine in the presence of 10 μM of the indicated compounds. Each enzyme activity was compared with that in the absence of inhibitors (8.19 nmol/min per mg of protein) and % inhibition is given. Mean values ± S.D. are shown ($n = 3$).

acid and ethanolamine as described previously [23]. RBL-1 (rat basophilic leukaemia-1) cells were kindly provided by Dr Shuh Narumiya (Kyoto University, Kyoto, Japan).

Syntheses of inhibitors

Hexadecanoic acid, cyclohexyl ester (compound **1**)

The synthesis of hexadecanoic acid, cyclohexyl ester (compound **1** in Figure 1) was as described previously by Vandevoorde et al. [22].

(±)-Hexadecanoic acid, 2-methylcyclohexyl ester (**2**)

In a two-neck flask, 2.41 ml (19.64 mmol) of 2-methylcyclohexanol (mixture of *cis* and *trans*) was poured into 10 ml of dry methylene chloride. The solution was cooled in an ice bath and magnetically stirred. Palmitoyl chloride (1 ml, 3.27 mmol) was added dropwise. The reaction mixture was stirred for 2 h at room temperature and then washed with 1 M HCl, 5 % bicarbonate solution and brine. The organic layer was dried over $MgSO_4$ and the solvent was evaporated under reduced pressure to give 0.9 g

(82 %) of colourless oil. TLC (ethyl acetate/methanol, 8:2, v/v) $R_F = 0.83$; ^{1}H NMR (C^2HCl_3) δ 0.88–0.92 (m, 6 H), 1.1–1.4 (m, 26 H), 1.5–1.9 (m, 9 H), 2.3 (t, *J* 7 Hz, 2 H), 4.81–5.0 (m, 1 H); ^{13}C NMR (C^2HCl_3) δ 14.10, 17.27 (CH_3), 18.43, 20.77, 22.71, 24.58, 25.29, 28.92, 29.24, 31.83, 33.58, 34.68, 35.58, 35.97, 37.33, 40.37 (CH_2), 73.49 (CH), 173.58 (C=O); IR ν (cm^{-1}) 1734 (C=O); CAS number 97635-30-8.

(±)-Hexadecanoic acid, 3-methylcyclohexyl ester (**3**)

The procedure described for compound **2** was used, with 2.45 ml (19.64 mmol) of 3-methylcyclohexanol (mixture of *cis* and *trans*) and 1 ml (3.27 mmol) of palmitoyl chloride to give 0.88 g (76 %) of colourless oil. TLC (ethyl acetate/methanol, 8:2, v/v) $R_F = 0.82$; ^{1}H NMR (C^2HCl_3) δ 0.83–0.90 (m, 6 H), 1.22–1.28 (m, 26 H), 1.62–1.88 (m, 9 H), 2.24 (t, *J* 7 Hz, 2 H), 4.73–4.92 (m, 1 H); ^{13}C NMR (C^2HCl_3) δ 14.10, 18.64 (CH_3), 20.05, 21.99, 22.12, 22.38, 24.26, 25.16, 26.59, 29.37, 31.38, 33.19, 34.22, 35.52, 40.63, 41.66 (CH_2), 70.07 (CH), 173.39 (C=O); IR ν (cm^{-1}) 1734 (C=O); analysis ($C_{23}H_{44}O_2$) C, H, N.

(±)-Hexadecanoic acid, 4-methylcyclohexyl ester (**4**)

The procedure described for compound **2** was used, with 2.46 ml (19.64 mmol) of 4-methylcyclohexanol (mixture of *cis* and *trans*) and 1 ml (3.27 mmol) of palmitoyl chloride to give 0.97 g (84 %) of a white solid. TLC (ethyl acetate/methanol, 8:2, v/v) $R_F = 0.88$. ^{1}H NMR (C^2HCl_3) δ 0.87–0.92 (m, 6 H), 1.15–1.48 (m, 26 H), 1.71–1.95 (m, 9 H), 2.27 (t, *J* 7 Hz, 2 H), 4.71–4.84 (m, 1 H); ^{13}C NMR (C^2HCl_3) δ 14.10, 16.58 (CH_3), 21.67, 21.93, 22.70, 25.17, 29.18, 29.31, 29.69, 31.25, 31.76, 32.28, 33.13, 33.44, 34.80, 35.71 (CH_2), 69.55 (CH), 173.39 (C=O); IR ν (cm^{-1}) 1731 (C=O); analysis ($C_{23}H_{44}O_2$) C, H, N.

Hexadecanoic acid, phenyl ester (**5**)

The procedure described for compound **2** was used, with 1.85 g (19.6 mmol) of phenol and 1 ml (3.27 mmol) of palmitoyl chloride to give 0.88 g (81 %) of a white solid. Melting point 44–45 °C (uncorrected); ^{1}H NMR (C^2HCl_3) δ 0.86 (t, *J* 3 Hz, 3 H), 1.15–1.46 (m, 26 H), 2.54 (t, *J* 3 Hz, 2 H), 7.05–7.08 (m, 2 H), 7.21–7.23 (m, 1 H), 7.34–7.39 (m, 2 H); ^{13}C NMR (C^2HCl_3) δ 14.35 (CH_3), 22.96, 25.22, 29.36, 29.49, 29.62, 29.95, 32.15, 34.67 (CH_2), 121.82, 125.90, 129.58 (CH), 151.13 (C), 172.48 (C=O); IR ν (cm^{-1}) 1732 (C=O); CAS number 24632-92-6.

Hexadecanoic acid, 2-methylphenyl ester (**6**)

The procedure described for compound **2** was used, with 0.68 ml (6.55 mmol) of *o*-cresol, 0.5 ml (3.59 mmol) of triethylamine and 1 ml (3.27 mmol) of palmitoyl chloride to give 0.88 g (78 %) of a brown solid. Melting point 34–36 °C (uncorrected); TLC (chloroform/methanol, 8:2, v/v) $R_F = 0.75$; ^{1}H NMR (C^2HCl_3) δ 0.88 (t, *J* 3 Hz, 3 H), 1.14–1.48 (m, 26 H), 2.17 (s, 3 H), 2.57 (t, *J* 7 Hz, 2 H), 6.97–7.20 (m, 4 H); ^{13}C NMR (C^2HCl_3) δ 14.74, 16.88 (CH_3), 23.42, 25.81, 29.95, 30.40, 32.60, 34.99, 46.57 (CH_2), 122.60, 126.54, 127.58, 130.81 (CH), 131.78, 150.16 (C), 172.67 (C=O); IR ν (cm^{-1}) 1755 (C=O); CAS number 4907-60-2.

Hexadecanoic acid, 3-methylphenyl ester (**7**)

The procedure described for compound **2** was used, with 0.7 ml (10.55 mmol) of *m*-cresol, 0.5 ml (3.59 mmol) of triethylamine and 1 ml (3.27 mmol) of palmitoyl chloride to give 0.90 g (80 %) of a light yellowish solid. Melting point 38–39 °C (uncorrected); TLC (chloroform/methanol, 8:2, v/v) $R_F = 0.79$; ^{1}H NMR (C^2HCl_3) δ 0.90 (t, *J* 3 Hz, 3 H), 1.22–1.51 (m, 26 H), 2.38 (s, 3 H), 2.56 (t, *J* 7 Hz, 2 H), 6.88–7.30 (m, 4 H); ^{13}C NMR (C^2HCl_3) δ 14.10, 21.28 (CH_3), 22.71, 25.03, 29.11, 29.37, 29.50, 29.69, 31.96, 34.48 (CH_2), 118.53, 122.15, 126.48, 129.07 (CH), 139.55, 150.82 (C), 172.36 (C=O); IR ν (cm^{-1}) 1751 (C=O); CAS number 4907-36-2.

Hexadecanoic acid, 4-methylphenyl ester (**8**)

The procedure described for compound **2** was used, with 0.68 ml (6.55 mmol) of *p*-cresol, 0.5 ml (3.59 mmol) of triethylamine and 1 ml (3.27 mmol) of palmitoyl chloride to give 0.92 g (81 %) of a white solid. Melting point 47–48 °C (uncorrected); TLC (chloroform/methanol, 8:2, v/v) $R_F = 0.79$; ^{1}H NMR (C^2HCl_3) δ 0.91 (t, *J* 3 Hz, 3 H), 1.12–1.51 (m, 26 H), 2.38 (s, 3 H), 2.56 (t, *J* 7 Hz, 2 H), 7.00 (d, *J* 8 Hz, 2 H); 7.14 (d, *J* 8 Hz, 2 H); ^{13}C NMR (C^2HCl_3) δ 15.19, 21.92 (CH_3), 23.80, 26.06, 30.21, 30.33, 30.46, 30.79, 33.05, 35.51 (CH_2), 122.34, 130.94 (CH), 136.32, 149.71 (C), 173.52 (C=O); IR ν (cm^{-1}) 1748 (C=O); CAS number 4907-37-7.

Hexadecanoic acid, 4-bromophenyl ester (**9**)

The procedure described for compound **2** was used, with 1.13 g (6.55 mmol) of 4-bromophenol, 0.5 ml (3.59 mmol) of triethylamine and 1 ml (3.27 mmol) of palmitoyl chloride to give 1.02 g (76 %) of a white solid. Melting point 56–57 °C (uncorrected); ^{1}H NMR (C^2HCl_3) δ 0.89 (t, *J* 5 Hz, 3 H), 1.09–1.29 (m, 26 H), 2.56 (t, *J* 7 Hz, 2 H), 6.98 (d, *J* 8 Hz, 2 H); 7.47 (d, *J* 8 Hz, 2 H); ^{13}C NMR (C^2HCl_3) δ 14.50 (CH_3), 23.04, 25.30, 29.51, 29.63, 29.83, 30.03, 32.29, 34.75 (CH_2), 119.12, 132.77 (CH), 123.77, 150.24 (C), 172.24 (C=O); IR ν (cm^{-1}) 1745 (C=O); CAS number 99053-00-6.

Hexadecanoic acid, 4-nitrophenyl ester (**10**)

The procedure described for compound **2** was used, with 0.91 g (6.55 mmol) of 4-nitrophenol, 0.5 ml (3.59 mmol) of triethylamine and 1 ml (3.27 mmol) of palmitoyl chloride to give 0.91 g (74 %) of a white solid. Melting point 63–64 °C (uncorrected); TLC (hexane/ethyl acetate, 9:1, v/v) $R_F = 0.85$; ^{1}H NMR (C^2HCl_3) δ 0.94 (t, *J* 7 Hz, 3 H), 1.21–1.52 (m, 26 H), 2.66 (t, *J* 7 Hz, 2 H), 7.33 (d, *J* 8 Hz, 2 H); 8.32 (d, *J* 8 Hz, 2 H); ^{13}C NMR (C^2HCl_3) δ 14.10 (CH_3), 22.71, 24.84, 29.11, 29.50, 29.69, 31.96, 34.42 (CH_2), 122.47, 125.19 (CH), 145.38, 155.66 (C), 171.26 (C=O); IR ν (cm^{-1}) 1753 (C=O); CAS number 1492-30-4.

Hexadecanoic acid, 3-nitro-4-methylphenyl ester (**11**)

The procedure described for compound **2** was used, with 0.50 g (6.55 mmol) of 3-nitro-*p*-cresol, 0.5 ml (3.59 mmol) of triethylamine and 1 ml (3.27 mmol) of palmitoyl chloride to give 1.02 g (80 %) of a yellow solid. Melting point 47–48 °C (uncorrected); TLC (hexane/ethyl acetate, 9:1, v/v) $R_F = 0.84$; ^{1}H NMR (C^2HCl_3) δ 0.88 (t, *J* 7 Hz, 3 H), 1.26–1.34 (m, 26 H), 2.59 (t, *J* 7 Hz, 2 H), 5.32 (s, 3 H), 7.27 (d, *J* 6 Hz, 2 H); 7.32 (d, *J* 6 Hz, 2 H); 7.76 (s, 1 H); ^{13}C NMR (C^2HCl_3) δ 15.13, 21.08 (CH_3), 23.74, 25.87, 30.14, 30.27, 30.40, 30.72, 32.99, 35.32, 46.96 (CH_2), 119.23, 127.58, 134.44 (CH), 131.98, 148.8, 150.03 (C), 172.80 (C=O); IR ν (cm^{-1}) 1768 (C=O); analysis ($C_{23}H_{37}NO_4$) C, H, N.

1-Hexadecanol, benzoate (**12**)

The procedure described for compound **2** was used, with 1.5 g (6.19 mmol) of 1-hexadecanol, 0.9 ml (6.46 mmol) of triethylamine and 1.44 ml (12.37 mmol) of benzoyl chloride to give 1.69 g (79 %) of a yellow solid. Melting point 30–31 °C (uncorrected); ^{1}H NMR (C^2HCl_3) δ 0.87 (t, J 7 Hz, 3 H), 1.28–1.49 (m, 26 H), 4.31 (t, J 7 Hz, 2 H), 7.43 (t, J 7 Hz, 1 H); 7.50–7.67 (m, 2 H); 8.03–8.17 (m, 2 H); ^{13}C NMR (C^2HCl_3) δ 14.22 (CH_3), 22.83, 26.26, 28.92, 29.49, 29.88, 32.08, 46.12, 65.27 (CH_2), 128.48, 129.07, 129.72, 130.75, 134.70 (CH), 132.95 (C), 166.85 (C=O); IR ν (cm^{-1}) 1792 (C=O); CAS number 22485-54-7.

Cyclohexanecarbonylhexadecanol (**13**)

The procedure described for compound **2** was used, with 1.5 g (6.19 mmol) of 1-hexadecanol, 1.5 ml (10.8 mmol) of triethylamine and 1.66 ml (12.38 mmol) of cyclohexanecarbonyl chloride. The reaction mixture was stirred for 2 h at room temperature and then washed with 1 M HCl, 5 % bicarbonate solution and brine. The organic layer was dried over $MgSO_4$ and the solvent was evaporated under reduced pressure. The residue was chromatographed on silica gel (eluting with ethyl acetate/hexane, 1:19, v/v) to give 1.5 g (70 %) of colourless oil. TLC (ethyl acetate/hexane, 1:19, v/v) $R_F = 0.72$; ^{1}H NMR (C^2HCl_3) δ 0.80 (t, J 11 Hz, 3 H), 1.03–1.22 (m, 28 H), 1.30–1.40 (m, 2 H), 1.50–1.55 (m, 2 H), 1.64–1.68 (m, 4 H), 1.79–1.83 (m, 2 H), 2.17–2.24 (m, 1 H), 3.97 (t, J 13 Hz, 2 H); ^{13}C NMR (C^2HCl_3) δ 14.10 (CH_3), 22.77, 25.55, 25.88, 26.00, 28.79, 29.11, 29.31, 29.43, 29.63, 29.76, 32.02, 43.34, 64.24 (CH_2), 176.11 (C=O); IR ν (cm^{-1}) 1735 (C=O); CAS number 500717-85-1.

N-Hexadecanoylaniline (**14**) and *N*-hexadecanoylcyclohexylamine (**15**)

The syntheses of compounds **14** and **15** were described previously by Vandevoorde et al. [22] and by Lambert et al. [24] respectively.

N-Benzoylpentadecylamine (**16**)

The procedure described for compound **2** was used, with 7.15 g (25.79 mmol) of 1-pentadecylamine, 0.5 ml (3.59 mmol) of triethylamine and 1.5 ml (12.89 mmol) of benzoyl chloride to give 3.29 g (77 %) of a white solid. Melting point 68–69 °C (uncorrected); ^{1}H NMR (C^2HCl_3) δ 0.91 (t, J 7 Hz, 3 H), 1.21–1.49 (m, 26 H), 3.49 (t, J 7 Hz, 2 H), 7.45–7.82 (m, 5 H); ^{13}C NMR (C^2HCl_3) δ 14.10 (CH_3), 22.71, 27.11, 29.37, 29.69, 31.96, 33.51, 40.17, 42.18 (CH_2), 126.87, 128.55 (CH), 131.27, 135.09 (C), 167.51 (C=O); IR ν (cm^{-1}) 1631 (C=O); analysis ($C_{22}H_{37}NO$) C, H, N.

N-Cyclohexanecarbonylpentadecylamine (**17**)

The procedure described for compound **2** was used, with 3.38 g (14.86 mmol) of 1-pentadecylamine, 0.5 ml (3.59 mmol) of triethylamine and 1 ml (7.43 mmol) of cyclohexanecarbonyl chloride to give 2 g (80 %) of a white solid. Melting point 75–76 °C (uncorrected); ^{1}H NMR (C^2HCl_3) δ 0.87 (t, J 7 Hz, 3 H), 1.22–1.43 (m, 26 H), 1.44–1.46 (m, 8 H), 2.66–2.71 (m, 1 H), 3.20–3.23 (m, 4 H); ^{13}C NMR (C^2HCl_3) δ 15.13 (CH_3), 23.74, 26.91, 28.01, 30.40, 32.99 (CH_2), 34.80 (CH), 40.43, 43.27, 46.77 (CH_2), 176.94 (C=O); IR ν (cm^{-1}) 1637 (C=O); analysis ($C_{22}H_{43}NO$) C, H, N.

Enzyme preparation

Wistar rats (250–500 g body weight; Charles River Japan, Yokohama, Japan) were anaesthetized with diethyl ether and killed by cervical dislocation. Lung and liver were removed and homogenized in 9 vol. (lung) or 5 vol. (liver) of ice-cold 20 mM Tris/HCl (pH 7.4) containing 0.32 M sucrose with a Polytron homogenizer.

The homogenates of lung were centrifuged at 800 ***g*** for 15 min and then at 12000 ***g*** for 30 min. The 12000 ***g*** pellet was suspended in PBS (pH 7.4) and subjected to two cycles of freezing and thawing. The sample was centrifuged at 105000 ***g*** for 50 min, and the resulting supernatant was used for the NPAA assay.

The homogenates of rat liver were centrifuged at 800 ***g*** for 15 min and then at 105000 ***g*** for 50 min. The 105000 ***g*** pellet was treated with 1 % Triton X-100 and again centrifuged at 105000 ***g*** for 50 min. The resulting supernatant was used for the FAAH assay.

Alveolar macrophages were obtained from bronchoalveolar lavage fluid of rat lung. RBL-1 cells were cultured as described previously [19]. The homogenates of macrophages and RBL-1 cells were prepared by sonic disruption for 3×3 s. Protein concentration was determined by the method of Bradford [25] with BSA as standard.

Enzyme assay

NPAA was allowed to react at 37 °C for 30 min with 100 μM *N*-[^{14}C]palmitoylethanolamine (10000 c.p.m., dissolved in 5 μl of DMSO) in 100 μl of 50 mM citrate/sodium phosphate at pH 5 containing the test compound (dissolved in 5 μl of DMSO/ethanol, 9:1, v/v), 0.1 % Triton X-100 and 3 mM DTT. The reaction was terminated by the addition of 0.35 ml of diethyl ether/methanol/1 M citric acid (30:4:1, by vol.). The ether extract (100 μl) was then spotted on a silica gel TLC plate (10 cm height) and developed at 4 °C for 20 min in chloroform/methanol/28 % ammonium hydroxide (80:20:2, by vol.). The distribution of radioactivity on the TLC plate was quantified using a Fujix bioimaging analyser BAS1500.

RESULTS

Screening of palmitic acid derivatives as NPAA inhibitors

NPAA from rat lung was allowed to react with *N*-[^{14}C]palmitoylethanolamine. The [^{14}C]palmitic acid produced was separated by TLC and quantified using a BAS1500 bioimaging analyser [20]. Using this assay method, cyclohexyl hexadecanoate (compound **1** in Figure 1) and other esters of palmitic acid (**2**–**11**) were tested at 10 μM for their inhibitory effects on NPAA. As shown in Figure 1, compound **1** inhibited the enzyme by 31 %, but the other ester compounds tested were less active or almost inactive, except for *p*-nitrophenyl hexadecanoate (**10**) and *p*-methyl-*m*-nitrophenyl hexadecanoate (**11**). We next tested compound **17**, in which the ester bond of compound **1** was changed to a 'retroamide' bond, and found that this compound was more active than compound **1**. It should be noted that compound **15**, in which the ester bond of compound **1** was changed to an amide bond, had a lower inhibitory effect than compound **1**. Another retroamide compound (**16**), in which the cyclohexanecarbonyl moiety of compound **17** was replaced by a benzoyl moiety, also showed potent inhibitory activity. In our previous study [22], 'retroesters' of palmitic acid (hexadecyl acetate and hexadecyl propionate) were shown to be moderate inhibitors of NPAA. However,

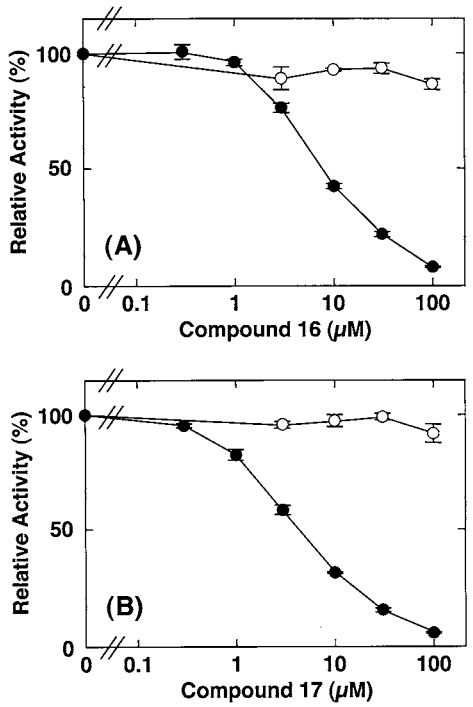

Figure 2 Concentration-dependent inhibition of NPAA by compounds 16 and 17

Rat lung NPAA (11.7 μg of protein; ●) or rat liver FAAH (110 μg of protein; ○) was allowed to react with 100 μM *N*-[^{14}C]palmitoylethanolamine in the presence of increasing concentrations of compound **16** (**A**) or **17** (**B**). A reaction mixture containing 50 mM citrate/sodium phosphate (pH 5), 0.1 % Triton X-100 and 3 mM DTT was used for both the NPAA and FAAH assays. The activities in the absence of inhibitors (8.16 nmol/min per mg of protein for NPAA and 0.53 nmol/min per mg of protein for FAAH) were normalized to 100 %. Mean values ± S.D. are shown ($n = 3$).

retroesters **12** and **13**, which have benzoyl and cyclohexanecarbonyl moieties respectively, were almost inactive.

Characterization of compounds 16 and 17 as NPAA inhibitors

Compounds **16** and **17** inhibited NPAA dose-dependently (Figure 2). Their IC_{50} values were calculated to be 8.3 and 4.5 μM respectively. Compounds **16** and **17** at 100 μM inhibited NPAA by 93 % and 95 % respectively. Both compounds were also tested for their inhibitory effects on rat liver FAAH. Although FAAH is known to be most active at pH 8.5–10 [11], in the experiments of Figure 2 we performed the FAAH assay at pH 5 rather than at alkaline pH, since we considered that the change in pH might influence the inhibitory effect of these compounds. Likewise, 3 mM DTT and 0.1 % Triton X-100, which were used as activators for NPAA, were also included in the reaction mixture for the FAAH assay. Under these assay conditions, FAAH was insensitive to compounds **16** and **17**, at least up to 100 μM. Also, when tested at pH 9, neither of the two compounds inhibited FAAH up to 100 μM (results not shown).

The manner of inhibition by compound **17** was examined by incubating different concentrations of *N*-palmitoylethanolamine with NPAA in the presence of 5 μM compound **17** or in its absence (Figure 3). Judging from the results shown in the Lineweaver–Burk plot, the inhibition was of a non-competitive type. Irrespective of the presence or absence of compound **17**, the apparent K_m value for *N*-palmitoylethanolamine was approx. 50 μM. We also examined the reversibility of the inhibition of

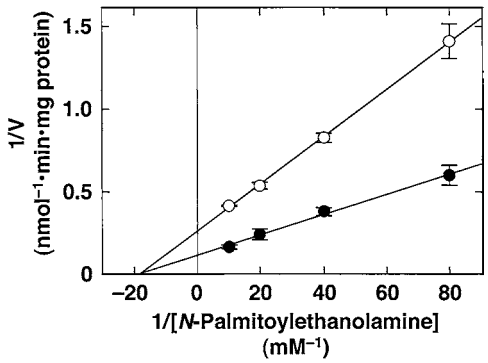

Figure 3 Non-competitive inhibition of NPAA by compound 17

Rat lung NPAA (6.69 μg of protein) was allowed to react with the indicated concentrations of *N*-[^{14}C]palmitoylethanolamine in the presence of 5 μM compound **17** (○) or in its absence (●). The values (means ± S.D., $n = 3$) are shown in a Lineweaver–Burk plot.

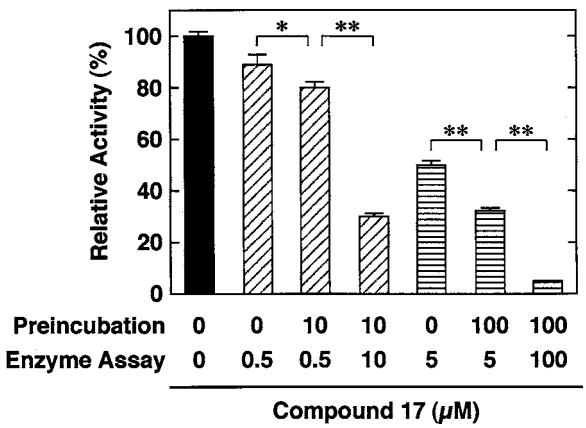

Figure 4 Reversibility of the inhibition of NPAA by compound 17

Rat lung NPAA (10.6 μg of protein) was preincubated with the indicated concentrations of compound **17** at room temperature for 15 min. After 20-fold dilution, the enzyme was subjected to assay in the presence of the indicated concentrations of compound **17**. Mean values ± S.D. are shown ($n = 3$); $^*P < 0.05$ and $^{**}P < 0.01$ by one-way ANOVA followed by Student's *t* test.

NPAA by compound **17** (Figure 4). NPAA was preincubated with compound **17**, and then diluted 20-fold before the reaction was initiated by the addition of *N*-palmitoylethanolamine. The results showed that the inhibitory activity of compound **17** was determined mostly by the diluted concentration of compound **17** (actual concentration during the enzyme assay) rather than by its initial concentration (concentration during the preincubation), suggesting that the inhibition is reversible.

Compound 17 as a biochemical tool to distinguish NPAA from FAAH

Next, we examined whether compound **17** could act as an NPAA inhibitor in intact cells expressing NPAA. Rat alveolar macrophages, having a high activity of NPAA [20], were used for this purpose. Since the FAAH activity in these cells has not been examined, we first estimated both FAAH and NPAA activities with homogenates of alveolar macrophages (Figure 5). When the homogenates were allowed to react with *N*-palmitoylethanolamine at pH 5 (the optimal pH for NPAA) and at pH 9 (the optimal pH for FAAH), a high *N*-palmitoylethanolamine-hydrolysing activity (specific activity 5.24 nmol/min per mg of protein) was observed at pH 5. In contrast, only a trace activity

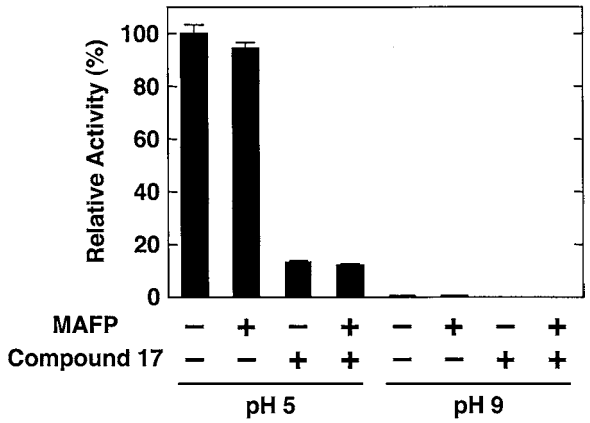

Figure 5 Inhibitory effect of compound 17 on *N*-palmitoylethanolamine hydrolysis in macrophage homogenates

Homogenates of rat alveolar macrophages (14.5 μg of protein) were allowed to react with 100 μM *N*-[^{14}C]palmitoylethanolamine either in 50 mM citrate/sodium phosphate (pH 5) containing 0.1 % Triton X-100 and 3 mM DTT or in 50 mM Tris/HCl (pH 9). MAFP at 1 μM, compound **17** at 100 μM or both were also included as indicated. The activity in the absence of inhibitors (5.24 nmol/min per mg of protein) was expressed as 100 %. Mean values $\pm$ S.D. are shown ($n = 3$).

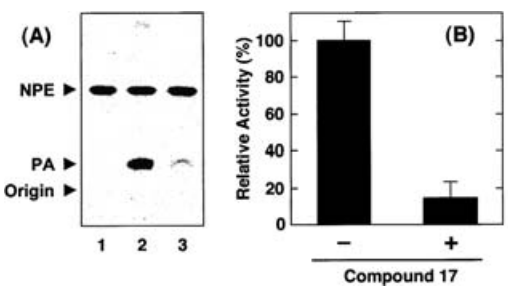

Figure 6 Inhibitory effect of compound 17 on NPAA in intact macrophages

Intact rat alveolar macrophages (2×10^7 cells) were suspended in 0.2 ml of RPMI 1640, and were preincubated in the presence of 200 μM compound **17** or in its absence at 37 °C for 60 min. The cells were then allowed to react with 25 μM *N*-[^{14}C]palmitoylethanolamine (5000 c.p.m.) at 37 °C for 30 min. (**A**) A thin-layer chromatogram is shown. Medium alone (lane 1), macrophages without compound **17** (lane 2) and macrophages with compound **17** (lane 3) were subjected to the enzyme assay. Abbreviations: NPE, *N*-palmitoylethanolamine; PA, palmitic acid. (**B**) Relative activities (mean values $\pm$ S.D., $n = 4$) are shown. The activity in the absence of compound **17** (37 pmol/min) was expressed as 100 %.

was detected at pH 9. The activity at pH 5 was largely inhibited by 100 μM compound **17**, but was barely affected by 1 μM MAFP, a potent FAAH inhibitor [26,27]. These results with the homogenates showed that almost all of the *N*-palmitoylethanolamine-hydrolysing activity in rat alveolar macrophages was derived from NPAA rather than FAAH. As shown in Figure 6, when intact alveolar macrophages were pretreated with compound **17** for 60 min, the *N*-palmitoylethanolamine-hydrolysing activity was inhibited by 85 %. Thus compound **17** could behave as an NPAA inhibitor in intact cells.

We tried to distinguish NPAA from FAAH by the use of compound **17**. RBL-1 cells were reported previously to have FAAH activity [28], but the presence of NPAA in these cells has not been examined. Homogenates of RBL-1 cells were subjected to freezing and thawing, and centrifuged at 105 000 ***g***. The resultant supernatant (cytosol) and pellet (particulate fraction) were assayed for *N*-palmitoylethanolamine-hydrolysing activity at pH 5 and at pH 9 (Figure 7). When the reaction was performed at pH 5, the cytosol showed a higher activity than the particulate fraction. This activity of the cytosol was inhibited by 100 μM compound **17**, but not by 1 μM MAFP. In contrast, the particulate fraction was more active than the cytosol at pH 9, and the reaction was inhibited by MAFP, but not by compound **17**. These results are in agreement with the intracellular localization of FAAH, which was membrane-bound, and NPAA, which could be solubilized by freezing and thawing, and revealed that RBL-1 cells possess not only FAAH but also NPAA. Thus compound **17** could be utilized to detect NPAA even in cells expressing FAAH.

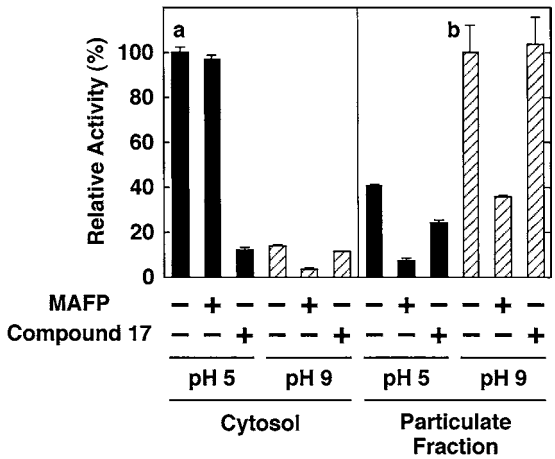

Figure 7 Detection of NPAA and FAAH in RBL-1 cells

Homogenates of RBL-1 cells were subjected to freezing and thawing, and then centrifuged at 105 000 ***g*** for 50 min. The supernatant (cytosol; 211 μg of protein) and the pellet (particulate fraction; 139 μg of protein) were allowed to react with 100 μM *N*-[^{14}C]palmitoylethanolamine either in 50 mM citrate/sodium phosphate (pH 5) containing 0.1 % Triton X-100 and 3 mM DTT or in 50 mM Tris/HCl (pH 9). MAFP at 1 μM or compound **17** at 100 μM was also included as indicated. Mean values $\pm$ S.D. are shown ($n = 3$). Activities in the absence of inhibitor (**a**, 0.18 nmol/min per mg of protein; **b**, 0.27 nmol/min per mg of protein) were expressed as 100 %.

DISCUSSION

N-Acylethanolamines, including anandamide and *N*-palmitoylethanolamine, are inactivated by enzymic hydrolysis to their corresponding fatty acids and ethanolamine. The enzymes responsible for this reaction reported so far are FAAH and NPAA [20]. The two enzymes show different substrate specificities in terms of *N*-acyl species of *N*-acylethanolamines. Namely, FAAH is the most active with anandamide, while NPAA hydrolyses *N*-palmitoylethanolamine at the highest rate [20]. The optimal pH is also different for these enzymes. The fact that NPAA acts only at acidic pH suggested its localization in lysosomes [20]. Furthermore, the organ distributions differ considerably. Rat brain and liver were rich sources of FAAH, but showed low NPAA activities [20]. In contrast, in the present study we revealed that the *N*-palmitoylethanolamine-hydrolysing activity of rat alveolar macrophages was derived almost exclusively from NPAA rather than FAAH. These differences in substrate specificity, intracellular localization and tissue distribution strongly suggest different physiological roles of FAAH and NPAA. FAAH is thought to play a central role in the regulation of brain levels of anandamide and other *N*-acylethanolamines as well as oleamide, based on studies using FAAH-deficient mice [16–18]. On the other hand, the physiological importance of NPAA remains unclear. Therefore selective NPAA inhibitors should be useful in elucidating the significance of NPAA.

In our initial studies on the development of selective NPAA inhibitors, we identified three derivatives of palmitic acid [cyclohexyl hexadecanoate (**1**), hexadecyl propionate and *N*-(3-hydroxypropionyl)pentadecanamide] with inhibitory actions [22]. However, these inhibitors were not very potent, with IC_{50} values higher than 10 μM (19.0, 53.8 and 31.8 μM respectively). In the present studies we used compound **1** and phenyl hexadecanoate (compound **5**) as parent compounds, and tested their various derivatives for the ability to inhibit NPAA. Among them, compounds **16** (*N*-benzoylpentadecylamine) and **17** (*N*-cyclohexanecarbonylpentadecylamine), which are 'retroamide' analogues of compounds **5** and **1**, were found to be the most potent, with IC_{50} values of 8.3 and 4.5 μM respectively. Since the retroamide compound *N*-(3-hydroxypropionyl)pentadecanamide was found previously to be an NPAA inhibitor as described above [22], potent activity of compounds **16** and **17** with a retroamide bond seemed reasonable in terms of structure–activity relationship. Moreover, we showed that compound **17** was a non-competitive, reversible inhibitor.

Importantly, compounds **16** and **17** did not inhibit FAAH, at concentrations up to 100 μM. This is in agreement with the observation that 'reversed amide analogues' of *N*-acylethanolamines [29] or a retroamide, *N*-(3-hydroxypropionyl)pentadecanamide [22], did not act as FAAH inhibitors. Several ester compounds, including cyclohexyl hexadecanoate (compound **1**), showed weak inhibitory activity towards FAAH [22]. In relation to this fact, we should note that FAAH, but not NPAA, has high esterase activity with fatty acyl esters such as 2-arachidonoylglycerol and methyl arachidonate [20]. Thus some ester compounds used in the present study and our previous study [22] might function as substrates of FAAH.

Since compounds **16** and **17** inhibited NPAA by more than 90 % at 100 μM, the compounds at this concentration were expected to distinguish NPAA from FAAH. In fact, using compound **17**, we could detect NPAA activity in RBL-1 cells (Figure 7), even though FAAH was also expressed in this cell line, as reported previously [19,28]. This enzyme assay with RBL-1 cells showed that the combination of compound **17** and MAFP (a potent FAAH inhibitor) was very useful for simultaneous quantification of the FAAH and NPAA activities in a single sample.

Compound **17** could also inhibit NPAA in intact macrophages. Thus this inhibitor may be applicable to various cultured cells and animal models. In contrast with the cell-free samples, preincubation of the inhibitor with the intact cells was required to achieve full inhibition (results not shown). Since NPAA as well as FAAH is present in the cell, *N*-palmitoylethanolamine must enter the cell across the cellular membrane before its enzymic hydrolysis. The uptake process of anandamide has been extensively investigated, and the occurrence of facilitated diffusion has been postulated [30,31]. However, published evidence for this was first questioned by Patricelli and Cravatt [32], and later by Glaser et al. [33] on the basis of experimental data. On the other hand, the uptake process of *N*-palmitoylethanolamine is poorly understood. One study concluded that *N*-palmitoylethanolamine is transported into mouse Neuro-2a neuroblastoma and rat RBL-2H3 basophilic leukaemia cells both by passive diffusion and by facilitated transport which is pharmacologically distinguishable from anandamide uptake [34]. Hence further investigations will be required in oder to elucidate the mechanism of *N*-palmitoylethanolamine uptake in other mammalian cells, including macrophages.

In summary, compound **17** (*N*-cyclohexanecarbonylpentadecylamine) is a selective, reversible inhibitor of NPAA with an IC_{50} of 4.5 μM, and was active both with the cell-free enzyme and in intact cells. This inhibitor is a useful tool to distinguish NPAA from FAAH. We are currently developing more potent inhibitors for NPAA.

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