Bioorganic & Medicinal Chemistry 22 (2014) 1468–1478

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Design, synthesis, and biological evaluation of a series of piperazine ureas as fatty acid amide hydrolase inhibitors

Mitsunori Kono [a,*], Takahiro Matsumoto [a], Toshihiro Imaeda [a], Toru Kawamura [a], Shinji Fujimoto [a], Yohei Kosugi [a], Tomoyuki Odani [a], Yuji Shimizu [a], Hideki Matsui [a], Masato Shimojo [a], Masakuni Kori [b]

[a] Pharmaceutical Research Division, Takeda Pharmaceutical Company Ltd, 26-1, Muraokahigashi 2-Chome, Fujisawa, Kanagawa 251-8555, Japan
[b] CMC Center, Takeda Pharmaceutical Company Ltd, 17-85, Jusohonmachi 2-Chome, Yodogawa-ku, Osaka 532-8686, Japan

ARTICLE INFO

Article history:
Received 1 November 2013
Revised 5 December 2013
Accepted 7 December 2013
Available online 20 December 2013

Keywords:
Fatty acid amide hydrolase inhibitors
Arachidonoylethanolamide
Piperazine ureas
Neuropathic pain
Inflammatory pain

ABSTRACT

A series of piperazine ureas were designed, synthesized, and evaluated for their potential as novel orally efficacious fatty acid amide hydrolase (FAAH) inhibitors for the treatment of neuropathic and inflammatory pain. We carried out an optimization study of compound **5** to improve its in vitro FAAH inhibitory activity, and identified the 2-pyrimidinylpiperazine derivative **21d** with potent inhibitory activity, favorable DMPK profile and brain permeability. Compound **21d** showed robust and dose-dependent analgesic efficacy in animal models of both neuropathic and inflammatory pain.

1. Introduction

Fatty acid amide hydrolase (FAAH)[1–3] is a membrane-bound serine hydrolase that catalyzes the intracellular hydrolysis of several endogenous lipid amides[4–7] such as endogenous cannabinoid (EC), anandamide (arachidonoylethanolamide: AEA),[8–11] sleep-inducing substance oleamide,[12–15] appetite-suppressing agent oleoyl ethanolamide (OEA),[16] and anti-inflammatory compound palmitoyl ethanolamide (PEA).[17] FAAH tightly controls the signaling function of these lipid amides, which have several physiological effects.[18–22] FAAH knockout mice are viable and healthy, have highly increased endogenous levels of AEA in several brain regions, and show an analgesic phenotype in several animal models of neuropathic and inflammatory pain.[23–25]

The cannabinoid 1 (CB1) receptor is highly expressed in hippocampus, striatum, nigra, olfactory bulb, and cerebellum in the central nervous system, and suppressively controls the release of neurotransmitters from sensory nerve.[26–28] On the other hand, the cannabinoid 2 (CB2) receptor is highly expressed in immune organs such as spleen and implicated in the regulation of inflammatory and immune response.[29,30] A potential therapeutic approach utilizing the activation of the CB1 receptor has been recognized as an attractive and beneficial way to treat pain, as well as other central nervous system disorders. Although direct activation of the CB1 receptor with CB1 agonists would exert strong pharmacological effects, it also causes a variety of undesirable adverse effects such as sedation, dependence, cognitive impairment, psychosis, and affection of cardiovascular system by their systemic activation.[31,32] The CB1 and CB2 receptors are activated by AEA. FAAH inhibitors enhance the activation of CB receptors by blocking AEA degradation. However, FAAH inhibitors may offer site-specific increase of AEA in tissues in which ECs are being produced through physiological responses, suggesting that they exhibit the pharmacological effects with less adverse effects. Moreover, FAAH inhibitors may provide anti-inflammatory effects by suppressing the release of inflammatory chemical mediators through stimulation of the CB2 receptor in immune cells.

Various classes of FAAH inhibitors, including urea derivatives (e.g., PF-04457845[33] and JNJ-40355003[34]), carbamates (e.g., URB597[35] and SA-47[36]), and keto-heterocycles (e.g., OL-135[37]) have been reported (Fig. 1). Previously, we reported a series of thiadiazolylpiperazine urea derivatives including compound **1** and found that analogues such as **2**, having the 3,4-dimethylisoxazolyl as the terminal heterocycle, showed potent FAAH inhibitory

Abbreviations: FAAH, fatty acid amide hydrolase; EC, endogenous cannabinoid; AEA, arachidonoylethanolamide; CB1, cannabinoid 1; CB2, cannabinoid 2; SAR, Structure–activity relationship; DMPK, drug metabolism and pharmacokinetics; PK, pharmacokinetics; PD, pharmacodynamics; Troc, 2,2,2-trichloroethoxy carbonyl; CFA, complete Freund's adjuvant; SNI, sciatic nerve injury.

* Corresponding author. Tel.: +81 466 32 1108; fax: +81 466 29 4455.
E-mail address: mitsunori.kouno@takeda.com (M. Kono).

0968-0896/$ - see front matter © 2014 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.bmc.2013.12.023

activity (Fig. 2).[38] Further optimization of 3,4-dimethylisoxazole compound **2** led to the identification of the thiazole derivative **3**, which orally showed anti-nociceptive effects in the acetic acid-induced writhing test in mice. In addition, we also found that compound **4**, which have the pyridazinyl group as the terminal heterocycle, possessed potent in vitro activity. In order to identify additional series of novel potent FAAH inhibitors, we focused on the modification of compound **4**. Our structure–activity relationship (SAR) study for the 3,4-dimethylisoxazole compounds[38] suggested that the urea moiety (**A** in Fig. 2) is essential for in vitro activity by interacting with catalytic residues Ser241 and Ser217, and that the piperazine moiety (**B** in Fig. 2) is also important as a central spacer. On the other hand, replacement of the thiadiazole ring with other 5-membered heterocycles was practical (**C** in Fig. 2). Taking into account this SAR information, we investigated a new series of compounds by modifying the ring structure of region **C** in compound **4**. In order to investigate the potential for a 6-membered ring in region C, we initially synthesized the pyrimidine derivative **5** and found the compound exhibited moderate FAAH inhibitory activity. In addition, replacement of the thiadiazole ring with a pyrimidine ring notably improved solubility (6.3 and 52 μg/mL in pH 6.8 solution for **4** and **5**, respectively). On the basis of these results, we selected compound **5** as a lead and performed an optimization study of compounds **X** to improve in vitro activity, focusing on the modification of the 6-membered ring (**Y**) and substituents (**R**) on the terminal benzene ring. Here, we describe the synthesis, SAR and biological evaluation of a series of novel piperazine-urea FAAH inhibitors having nitrogen-containing 6-membered heterocycles such as pyridine and pyrimidine rings.[39]

2. Chemistry

The general synthetic method for 2-pyrimidinylpiperazine and 4-pyrimidinylpiperazine derivatives (**5** and **21a**–**21e**) is shown in Scheme 1. The coupling reaction of 2,4-dichloropyrimidine **6** with *N*-Boc-piperazine gave a mixture of the 4-substituted compound **7** and 2-substituted compound **10** (**7**:**10** = ca. 12:1). These isomers were easily separated by silica gel column chromatography. Alternatively, compound **10** was regioselectively synthesized by the reaction of 2,4-dichloropyrimidine **6** with *tert*-butyl 4-methylpiperazine-1-carboxylate in 73% yield.[40] Suzuki coupling of **7** and **10** with appropriate aryl boronic acids provided compounds **8** and **11**–**15**. Acidic deprotection and subsequent urea formation with the corresponding 2,2,2-trichloroethoxy carbonyl (Troc) derivative **23**[41] or phenylcarbamate **24** yielded compounds **5** and **21a**–**21e**.

Compounds **44a**–**44e** were synthesized as shown in Scheme 2. Buchwald amination of aryl halide **25**–**27** with *N*-Boc-piperazine afforded corresponding aryl piperazine **29**–**31**. Suzuki coupling of compounds **28**–**31** with appropriate aryl boronic acids gave biaryl derivatives **34**–**36** and **38**, respectively. 4-Bromo-2-chloropyridine **32** was converted to biaryl **33** by Suzuki coupling, and subsequent Buchwald amination yielded compound **37**. Compounds **44a**–**44e** were obtained by acidic deprotection of **34**–**38** and subsequent urea formation with the Troc derivatives **23**.

3. Results and discussion

The synthesized compounds were evaluated using in vitro and in vivo biological tests as follows. FAAH inhibitory activity was measured by a fluorescence-based assay using human and rat FAAH enzyme fractions and a FAAH substrate, 7-amino-4-methylcoumarin arachidonoyl amide (AMCAA: manufactured by CAYMAN CHEMICAL) (Tables 1 and 2). The acetic acid-induced writhing test in mice was performed as the first screening of in vivo efficacy (Table 2). Further evaluation of analgesic efficacy of the selected compounds was conducted in the rat sciatic nerve injury (SNI)-induced neuropathic pain model and the rat complete Freund's adjuvant (CFA)-induced inflammatory pain model.

First, replacement of the pyrimidine of the ring **Y** with pyridine or rotation of the pyrimidine ring was examined. As shown in Table 1, pyridine derivatives **44a** and **44b**, in which the nitrogen atom at the 1-position (Y^1) or 3-position (Y^2) of the pyrimidine ring in compound **5** was replaced with CH, showed comparable inhibition to that of compound **5**. Interestingly, compound **44d** (Y^3 = N)

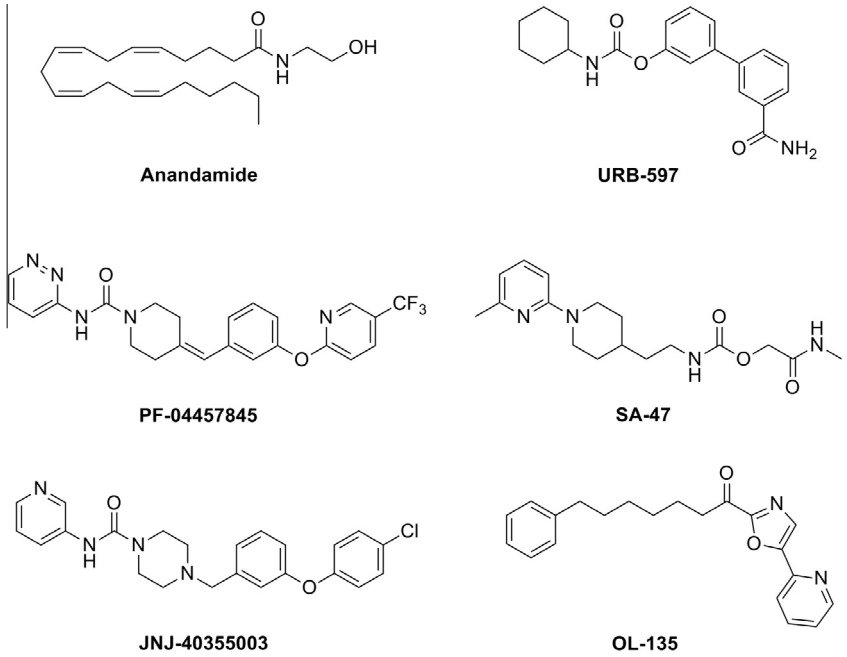


Figure 1. Anandamide (AEA) and known FAAH inhibitors.

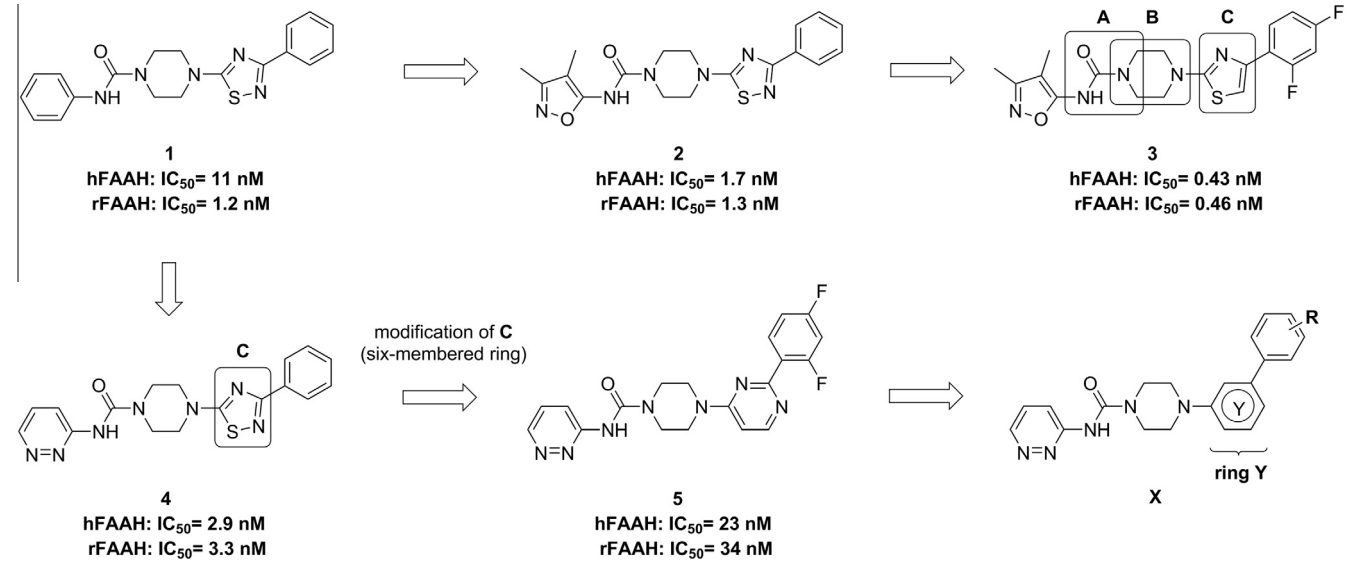
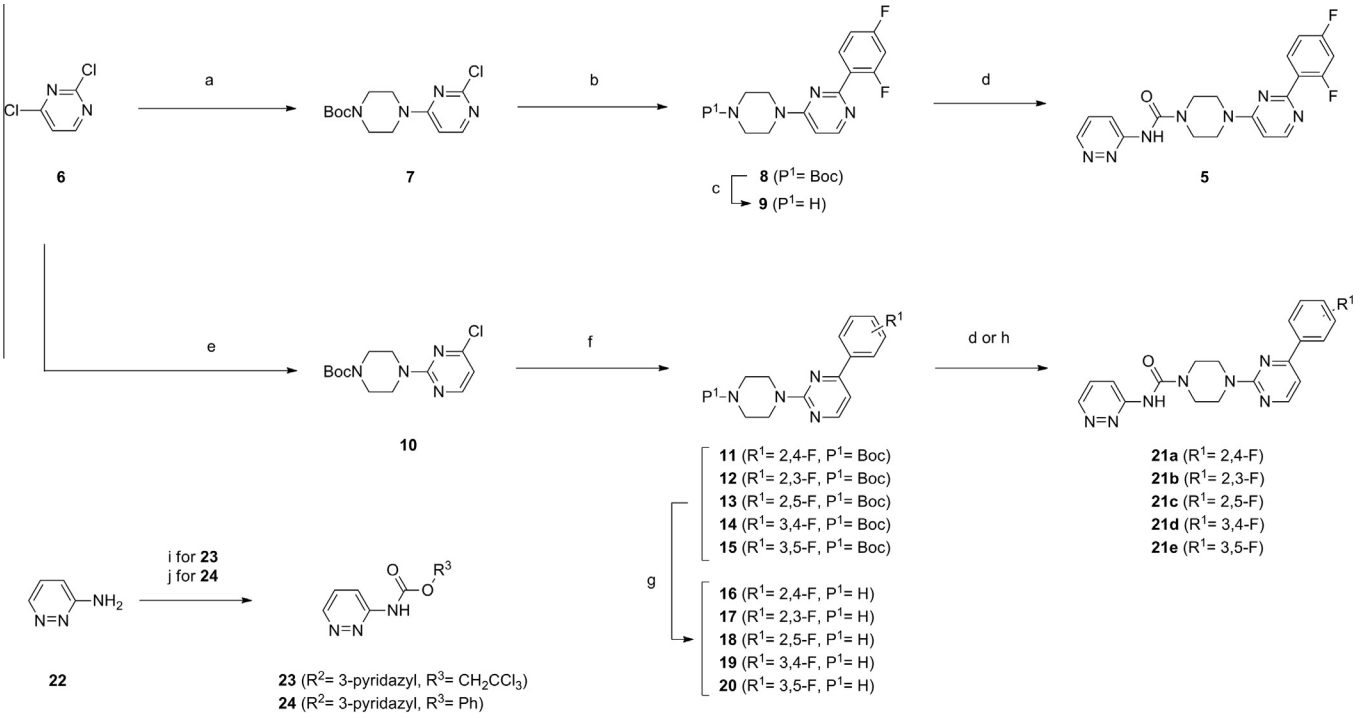


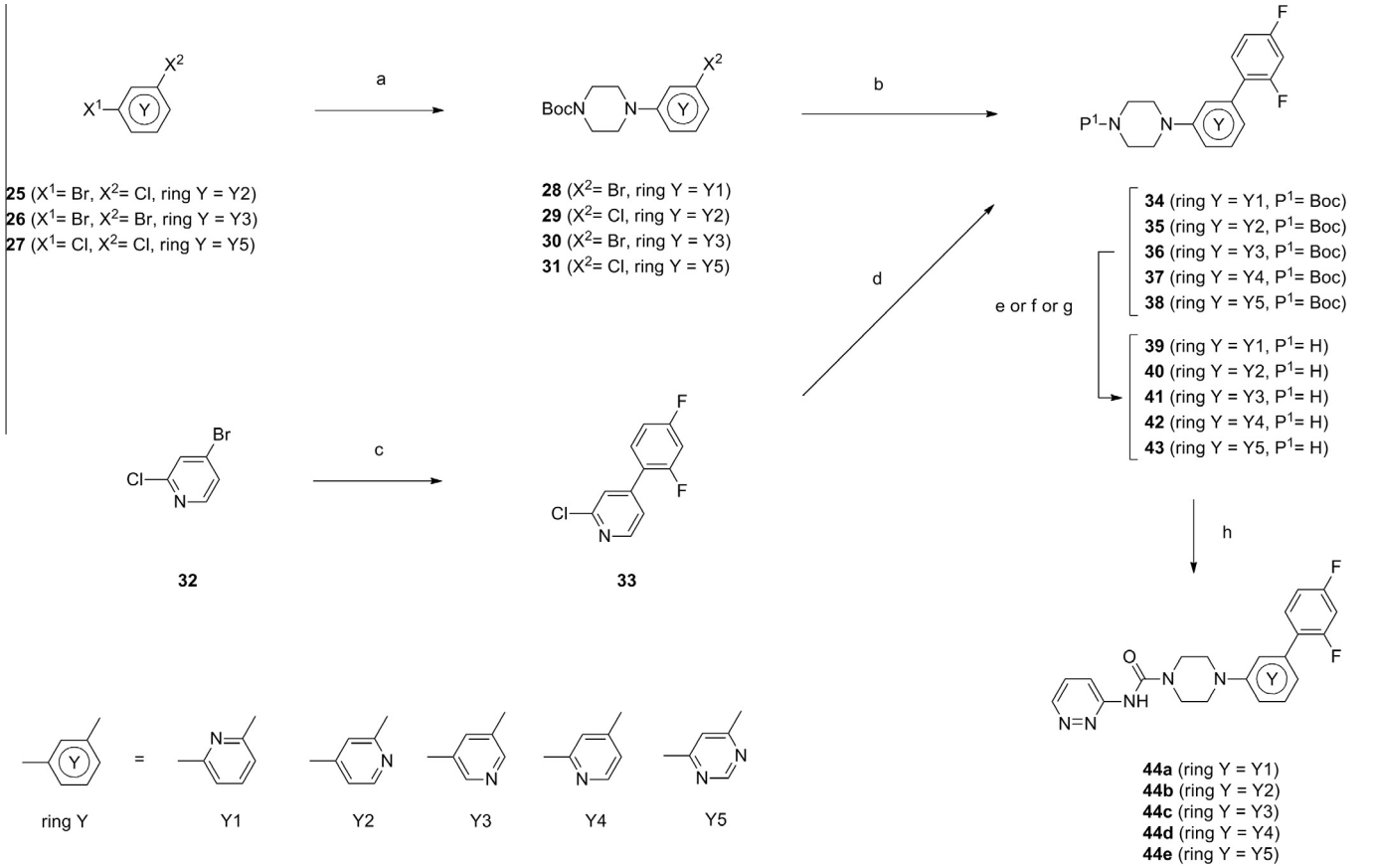
Figure 2. Structural modification of the hit compound **1**.



Scheme 1. Synthesis of compounds **5** and **21a**–**21e**. Reagents and Conditions: (a) *N*-Boc-piperazine, triethylamine, DMF, room temp, 90%; (b) (2,4-difluorophenyl)boronic acid, $Pd(PPh_3)_4$, 2 N aq Na_2CO_3, toluene, 100 °C, 40%; (c) 4 N HCl in EtOAc, room temp then 1 N NaOH, 85%; (d) **23**, $^iPr_2NEt$, DMSO, 70 °C, 22% [for **5** and **21a**]; (e) 1-Boc-4-methylpiperazine, toluene, 110 °C, 73%; (f) Ar-$B(OH)_2$, $Pd(PPh_3)_4$, 2 N aq Na_2CO_3, toluene or DME, 71%-quant.; (g) 4 N HCl in EtOAc, room temp., 80%-quant.; (h) **24**, Et_3N, acetone, 40 °C, 39–91% [for **21b**–**21e**]; (i) 2,2,2-trichloroethyl chloroformate, pyridine, THF, 0 °C, 46%; (j) phenylchloroformate, pyridine, MeCN, 0 °C, 87%.

exhibited more potent inhibitory activity for both human and rat FAAH, whereas compound **44c** (Y^4 = N) showed less potent activity. These results suggested that the nitrogen atom at Y^3 was important for FAAH inhibitory activity. Therefore, we synthesized pyrimidine derivatives, having the nitrogen atom at Y^3, and found that compounds **44e** and **21a** showed further increase in FAAH inhibitory activity.

Next, we investigated the optimal position of the difluoro groups of compound **21a**. The results are shown in Table 2. The 2,3-difluoro group (**21b**) enhanced human FAAH inhibitory activity, whereas rat FAAH inhibitory activity was not increased. The 2,5-difluoro and 3,5-difluoro derivatives (**21c** and **21e**) exhibited very potent activity for both human and rat FAAH. 3,4-Difluoro derivative **21d** was equipotent to compound **21a**. It was of our interest that the solubility of compound **21d** was notably improved (3.1 μg/mL in pH 6.8 solution) compared with that of other derivatives. In vivo analgesic efficacy of compounds **21b**–**21e** was evaluated using the acetic acid-induced writhing test in mice. Among them, compound **21d** exerted anti-nociceptive efficacy at a dose of 10 mg/kg, po. From these biological and physicochemical results, we selected compound **21d** for further evaluation.



Scheme 2. Synthesis of compounds **44a**–**44e**. Reagents and Conditions: (a) *N*-Boc-piperazine, $Pd_2(dba)_3$, xantphos, NaO^{t}Bu, toluene, 100 °C, 70–79%; (b) (2,4-difluorophenyl)boronic acid, $Pd(PPh_3)_4$, 2 N aq Na_2CO_3, toluene or DME, 37–94%; (c) 2,4-difluorophenylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, MeOH, 50 °C, 91%; (d) *N*-Boc-piperazine, $Pd(OAc)_2$, BINAP, NaO^{t}Bu, 1,4-dioxane, 85 °C, 76%; (e) TFA, CH_2Cl_2, room temp, 72% [for **39**]; (f) 4 N HCl in EtOAc, room temp then 1 N NaOH, 83–94% [for **40**, **41**, **43**]; (g) 4 N HCl in MeOH, room temp, 98% [for **42**]; (h) **23**, ^{i}Pr$_2$NEt, DMSO, 70 °C, 10–57%.

We examined the analgesic effects of compound **21d** in rat SNI-induced neuropathic pain model and CFA-induced inflammatory pain model (Fig. 3). In SNI rats, the pain threshold of the ipsilateral hind paw to tactile stimuli was dramatically decreased compared to that in sham-operated rats, indicating that SNI operation evoked tactile allodynia in rats. Orally administered compound **21d** at doses of 1–10 mg/kg significantly ameliorated tactile allodynia in a dose-dependent fashion in SNI rats. In CFA rats, the pain threshold of the ipsilateral hind paw to tactile stimuli was dramatically decreased compared to that in sham-operated rats, indicating that CFA injection evoked tactile allodynia in rats. Compound **21d** also significantly ameliorated tactile allodynia of the ipsilateral hind paw at doses of 3–10 mg/kg. In conclusion, these results in well-established pain models suggest that FAAH inhibitors such as compound **21d** have potential to improve abnormal pain involved in neuropathic and inflammatory pain in the clinic.

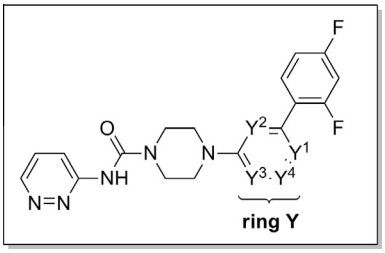
In order to confirm the pharmacokinetics (PK) and pharmacodynamics (PD) relationships, we measured the concentration of compound **21d** and AEA in plasma and brain after administration of the compound (0.03, 0.3, 3 mg/kg, po). Plasma and brain concentrations of compound **21d** were elevated in a dose-dependent manner (Table 3), consistent with the observed efficacy in rat pain models. Peak concentration levels of 803 ng/mL (plasma) and 1600 ng/g tissue (brain) were observed at 4 h after oral administration of compound **21d** at a dose of 3 mg/kg in rats, indicating that compound **21d** has high brain permeability (brain/plasma concentration ratio = 2.0). We also examined the effects on the tissue content of AEA as a PD marker for FAAH inhibition and found that the concentration of AEA in the brain was elevated at the efficacious dose of compound **21d**. These results indicate that compound **21d** exerted analgesic efficacy at doses where compound **21d** inhibited FAAH.

To date, two main classes of FAAH inhibitors with therapeutic potential have been reported. Urea and carbamate-type FAAH inhibitors show irreversible inhibition, and form a covalent tight-binding with the catalytic Ser-241 residue within the active site.[42] On the other hand, reversible inhibitors such as α-keto-heterocycles tend to form a tetrahedral intermediate arising from the interaction of the electrophilic carbonyl group with catalytic serine to give a hemiacetal bond.[43,44] Co-crystallization study of our thiadiazolylpiperazine urea derivative[38] revealed the compound forms a covalent bond with FAAH to inactivate the enzyme irreversibly. To investigate the inhibition mode of compound **21d**, we performed a progress curve analysis as an enzyme kinetic analysis (Fig. 4). Non-linear progress curve was observed indicating that the inhibitory activity of compound **21d** time-dependently increased. Taken together with previous structural analysis of piperazine-urea-type derivatives, the result suggested that compound **21d** probably forms a covalent bond with the active site Ser 241 of FAAH, which causes irreversible inhibition in a binding mode similar to that observed with the series of thiadiazole derivatives.

4. Conclusion

In the course of exploring novel FAAH inhibitors that are therapeutically effective against neuropathic and inflammatory pain, we

Table 1
Human and rat FAAH activities of derivatives modified at the thiadiazole ring moiety



Compound	Ring **Y**	Apparent FAAH IC_{50}[a] (nM) (human/rat)
5		23/34
44a		36/13
44b		28/12
44c		140/74
44d		2.2/4.3
44e		1.5/1.7
21a		1.1/0.43

[a] IC_{50} values were determined at a 30 min reaction.

designed, synthesized, and evaluated a series of piperazine urea compounds incorporating nitrogen-containing 6-membered heterocycles. Replacement of the 4-pyrimidinylpiperazine ring with a 2-pyrimidinylpiperazine led to an increase in FAAH inhibitory activity. The substitution pattern of the difluoro groups on the phenyl ring affected in vivo efficacy. We identified the 2-pyrimidinylpiperazine derivative **21d**, which dose-dependently ameliorated the decrease in pain threshold in animal models of both neuropathic and inflammatory pain. The results suggested that the potent and selective piperazine urea FAAH inhibitor **21d** would become a beneficial analgesic agent for the treatment of neuropathic and inflammatory pain.

5. Experimental section

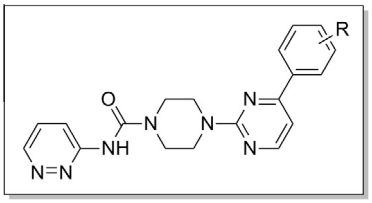
5.1. Chemistry

Melting points were determined with a Yanagimoto melting point apparatus or a Büchi melting point apparatus B-545 and are uncorrected. ^{1}H NMR spectra were obtained at 300 MHz on a Varian Ultra-300 or a Bruker DPX-300 spectrometer. Chemical shifts are given in δ values (ppm) using tetramethylsilane as the internal standard. Peak multiplicities are expressed as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublet; br, broad; br s, broad singlet; m, multiplet. Elemental analyses were carried out by Takeda Analytical Research Laboratories Ltd. Reactions were followed by TLC on Silica gel 60 F 254 precoated TLC plates (E. Merck) or NH TLC plates (Fuji Silysia Chemical Ltd). Chromatographic separations were carried out on silica gel 60 (0.063–0.200 or 0.040–0.063 mm, E. Merck) or basic silica gel (Chromatorex® NH, 100–200 mesh, Fuji Silysia Chemical Ltd) using the indicated eluents. Yields are unoptimized. The HPLC analyses were performed using a Shimadzu UFLC instrument. Elution was done with a gradient of 5–90% solvent B in solvent A (solvent A was 0.1% TFA in water, and solvent B was 0.1% TFA in acetonitrile) through a L-column 2 ODS (3.0 × 50 mm, 2 μm) column at 1.2 mL min^{-1}. Area % purity was measured at 254 nm.

5.1.1. *tert*-Butyl 4-(2-chloropyrimidin-4-yl)piperazine-1-carboxylate (7)

A mixture of 2,4-dichloropyrimidine (1.00 g, 6.71 mmol), *N*-Boc-piperazine (1.37 g, 7.38 mmol), triethylamine (1.40 mL, 10.1 mmol), and DMF (10 mL) was stirred at room temperature for 4.0 h. The mixture was diluted with water, and extracted with EtOAc. The organic layer was washed with water, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was triturated with Et_2O to give **7** (1.80 g, 90%) as a colorless powder.

Table 2
Human and rat FAAH inhibitory activities of derivatives modifying the central piperazine ring moiety



Compound	R	Apparent FAAH IC_{50}[a] (nM) (human/rat)	Solubility[b] (μg/mL)	Acetic acid writing test[c] % of inhibition
21a	2,4-F	1.1/0.43	0.85	N.T.
21b	2,3-F	0.072/1.2	0.16	18.9
21c	2,5-F	0.025/0.17	<0.06	2.2
21d	3,4-F	0.72/0.28	3.1	34.7**
21e	3,5-F	0.08/0.34	0.1	0.9

[a] IC_{50} values were determined at a 30 min reaction.
[b] Solubility in pH 6.8 solution.
[c] 10 mg/kg, po administration in mice.
** $p<0.01$ vs. vehicle-treated mice (Student's t-test).

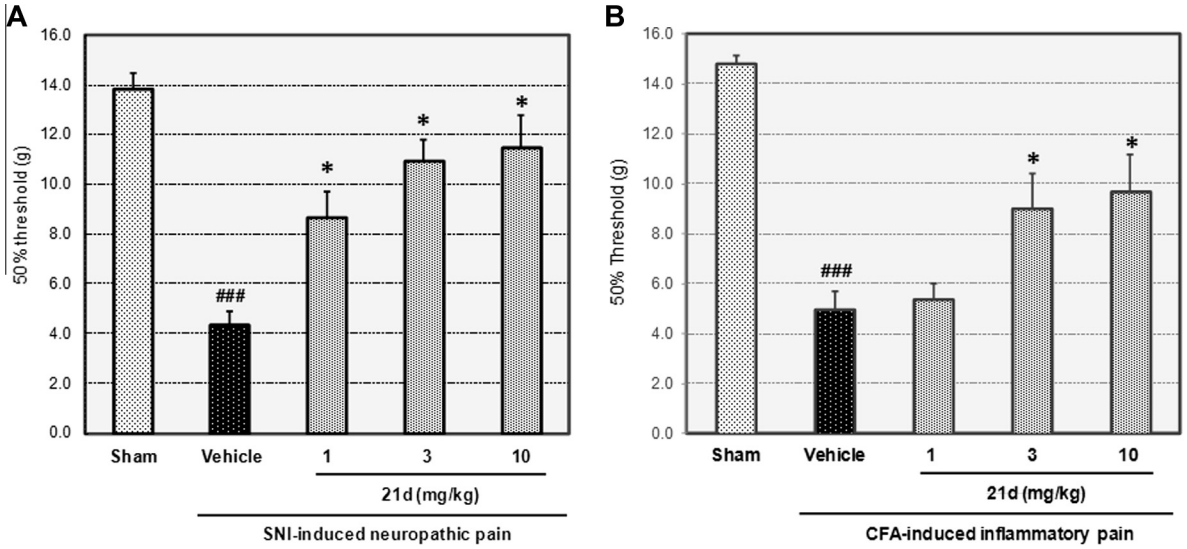


Figure 3. Analgesic effect of compound **21d** in rat neuropathic and inflammatory pain models. (A) Neuropathic pain was induced by sciatic nerve injury (SNI) in rats. Compound, **21d** and vehicle were orally administered 7 days after SNI operation. Tactile allodynia of ipsilateral hind paw was assessed by von Frey filaments 2 h after drug treatment. Data are shown in mean and SE of 8 rats. $^{###}p$ <0.001 versus sham-operated rats (Student's *t*-test). $^{*}p$ <0.025 versus vehicle-treated SNI rats (Shirley-Williams test). (B) Inflammatory pain was induced by CFA injection into hind paw. Compound **21d** and vehicle were orally administered 5 days after CFA injection. Tactile allodynia of ipsilateral hind paw was assessed by von Frey filaments 4 h after drug treatment. Data are shown in mean and SE of 8–10 rats. $^{###}p$ <0.001 versus sham-operated rats (Student's *t*-test). $^{*}p$ <0.025 versus vehicle-treated CFA rats (Shirley-Williams test).

Table 3
Pharmacokinetic and pharmacodynamic parameters in rats

	Dose	(mg/kg)	0.03		0.3		3	
			Plasma	Brain	Plasma	Brain	Plasma	Brain
Compound **21d**	C_{max}	(ng/mL or ng/g)	0	0	57	100	803	1600
	T_{max}	(h)	0	0	4.0	4.0	4.0	4.0
	AUC	(ng h/mL or ng h/g)	0	0	533	898	8819	15955
AEA	C_{max}	(ng/mL or ng/g)	0.2	6.8	0.4	19.1	0.5	19.2
	T_{max}	(h)	8.0	4.0	8.0	4.0	12.0	4.0
	AUC	(ng h/mL or ng h/g)	3.4	64.3	7.9	232.9	9.5	311.6

Mean (n = 3).
Compound **21d** was suspended in 0.5% methylcellulose solution for oral administration. The concentrations of compound **21d** and AEA in the plasma and brain were determined using liquid chromatography/tandem mass spectrometry (LC/MS/MS).

^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.49 (9H, s), 3.47–3.58 (4H, m), 3.60–3.71 (4H, m), 6.40 (1H, d, J = 6.2 Hz), 8.07 (1H, d, J = 6.2 Hz). MS (ESI): m/z 299 $[M+H]^+$.

5.1.2. *tert*-Butyl 4-[2-(2,4-difluorophenyl)pyrimidin-4-yl]piperazine-1-carboxylate (8)

A mixture of *tert*-butyl 4-(2-chloropyrimidin-4-yl)piperazine-1-carboxylate (1.80 g, 6.02 mmol), (2,4-difluorophenyl)boronic acid (1.43 g, 9.04 mmol), 1 M aq Na_2CO_3 (24 mL, 48.2 mmol), tetrakis(triphenylphosphine)palladium (0) (835 mg, 0.723 mmol), and toluene (60 mL) was stirred at 100 °C overnight under N_2 atmosphere. The mixture was diluted with water, and extracted with EtOAc. The organic layer was washed with water, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane–EtOAc) to give **8** (907 mg, 40%) as a colorless viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.49 (9H, s), 3.48–3.76 (8H, m), 6.44 (1H, d, J = 6.4 Hz), 6.84–6.99 (2H, m), 8.02–8.12 (1H, m), 8.36 (1H, d, J = 6.4 Hz).

5.1.3. 2-(2,4-Difluorophenyl)-4-(piperazin-1-yl)pyrimidine (9)

To a stirred solution of *tert*-butyl 4-[2-(2,4-difluorophenyl) pyrimidin-4-yl]piperazine-1-carboxylate (888 mg, 2.36 mmol) in EtOAc (9.0 mL) was added 4 M HCl in EtOAc (9.0 mL) dropwise at room temperature. After stirring at room temperature for 5.0 h, the mixture was concentrated in vacuo. The residue was dissolved in water, neutralized with 1 M NaOH, and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$, and concentrated in vacuo to give **9** (554 mg, 85%) as a pale yellow viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.01–3.17 (4H, m), 3.69–3.98

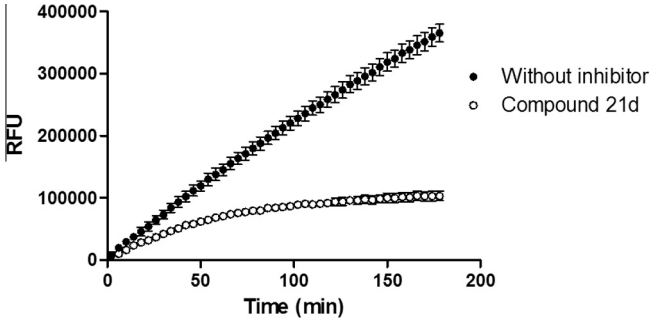


Figure 4. Enzyme kinetic analysis of compound **21d**. Kinetic analysis of FAAH inhibition by compound **21d**. Progress curve analysis was performed using AMCAA method at room temperature in the presence (○) or absence (●) of 10 nM compound **21d**. Data represent the mean ± S.E.M. from three independent experiments.

(4H, m), 6.37–6.52 (1H, m), 6.81–7.03 (2H, m), 7.93–8.17 (1H, m), 8.29–8.45 (1H, m).

5.1.4. 4-[2-(2,4-Difluorophenyl)pyrimidin-4-yl]-*N*-(pyridazin-3-yl)piperazine-1-carboxamide (5)

A mixture of 2-(2,4-difluorophenyl)-4-(piperazin-1-yl)pyrimidine (100 mg, 0.362 mmol), 2,2,2-trichloroethyl pyridazin-3-ylcarbamate (108 mg, 0.398 mmol), *N*-ethyldiisopropylamine (0.126 mL, 0.724 mmol), and DMSO (1.5 mL) was stirred at 70 °C overnight. The mixture was poured into water, and extracted with EtOAc. The organic layer was washed with water, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane–EtOAc), and recrystallized from EtOAc–hexane to give **5** (31.9 mg, 22%) as colorless crystals, mp 200–201 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.58–3.71 (4H, m), 3.72–3.84 (4H, m), 6.88 (1H, d, J = 6.2 Hz), 7.14–7.25 (1H, m), 7.28–7.39 (1H, m), 7.59 (1H, dd, J = 4.6, 9.1 Hz), 7.96–8.14 (2H, m), 8.36 (1H, d, J = 6.2 Hz), 8.85 (1H, d, J = 4.0 Hz), 9.99 (1H, s). MS (ESI): m/z 398 $[M+H]^+$ Anal. Calcd for $C_{19}H_{17}F_2N_7O\cdot0.5H_2O$: C, 56.15; H, 4.46; N, 24.13. Found: C, 56.08; H, 4.21; N, 24.00.

5.1.5. *tert*-Butyl 4-(4-chloropyrimidin-2-yl)piperazine-1-carboxylate (10)

A mixture of 2,4-dichloropyrimidine (50 g, 336 mmol), *tert*-butyl 4-methylpiperazine-1-carboxylate (67.2 g, 336 mmol), and toluene (500 mL) was stirred at 110 °C overnight. The mixture was poured into water, and extracted with EtOAc. The organic layer was washed with water, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane–EtOAc) to give **10** (109 g, 73%) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.49 (9H, s), 3.44–3.53 (4H, m), 3.75–3.84 (4H, m), 6.53 (1H, d, J = 5.1 Hz), 8.16 (1H, d, J = 5.1 Hz). MS (ESI): m/z 299 $[M+H]^+$.

5.1.6. *tert*-Butyl 4-[4-(2,4-difluorophenyl)pyrimidin-2-yl]piperazine-1-carboxylate (11)

Compound **11** was prepared in a manner similar to that described for **8** in 71% yield as a colorless viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.50 (9H, s), 3.46–3.58 (4H, m), 3.85–3.93 (4H, m), 6.83–7.08 (3H, m), 8.08–8.22 (1H, m), 8.39 (1H, d, J = 5.3 Hz). MS (ESI): m/z 377 $[M+H]^+$.

5.1.7. *tert*-Butyl 4-[4-(2,3-difluorophenyl)pyrimidin-2-yl]piperazine-1-carboxylate (12)

A mixture of *tert*-butyl 4-(2-chloropyrimidin-4-yl)piperazine-1-carboxylate (9.00 g, 30.1 mmol), (2,3-difluorophenyl)boronic acid (7.10 g, 45.1 mmol), 1 M aq Na_2CO_3 (41 mL, 82.0 mmol), tetrakis(triphenylphosphine)palladium (0) (4.2 g, 3.61 mmol), and DME (270 mL) was stirred at 95 °C overnight under N_2 atmosphere. After cooling to room temperature, the mixture was diluted with water, and extracted with EtOAc. The organic layer was washed with water, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane–EtOAc) to give **12** (11.3 g, quant.) as pale yellow crystals. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.50 (9H, s), 3.53 (4H, t, J = 4.0 Hz), 3.89 (4H, t, J = 4.0 Hz), 7.04–7.06 (1H, m), 7.16–7.29 (2H, m), 7.81–7.85 (1H, m), 8.41 (1H, d, J = 4.0 Hz). MS (ESI): m/z 377 $[M+H]^+$.

5.1.8. *tert*-Butyl 4-[4-(2,5-difluorophenyl)pyrimidin-2-yl]piperazine-1-carboxylate (13)

Compound **13** was prepared in a manner similar to that described for **12** in 96% yield as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.52 (9H, s), 3.53 (4H, t, J = 4.0 Hz), 3.88 (4H, t, J = 4.0 Hz), 7.09–7.14 (3H, m), 7.81–7.86 (1H, m), 8.41 (1H, d, J = 4.0 Hz). MS (ESI): m/z 377 $[M+H]^+$.

5.1.9. *tert*-Butyl 4-[4-(3,4-difluorophenyl)pyrimidin-2-yl]piperazine-1-carboxylate (14)

A mixture of *tert*-butyl 4-(2-chloropyrimidin-4-yl)piperazine-1-carboxylate (10.0 g, 33.5 mmol), (3,4-difluorophenyl)boronic acid (7.90 g, 50.2 mmol), 1 M aq Na_2CO_3 (45 mL, 90.0 mmol), tetrakis(triphenylphosphine)palladium (0) (4.6 g, 4.02 mmol), and DME (300 mL) was stirred at 95 °C overnight under N_2 atmosphere. After cooling to room temperature, the mixture was stirred at room temperature for 15 h, diluted with water, and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$, and concentrated in vacuo to give **14** as a brown oil. This product was used for next reaction without further purification.

5.1.10. *tert*-Butyl 4-[4-(3,5-difluorophenyl)pyrimidin-2-yl]piperazine-1-carboxylate (15)

Compound **15** was prepared in a manner similar to that described for **14** as a brown oil. This product was used for next reaction without further purification.

5.1.11. 4-(2,4-Difluorophenyl)-2-(piperazin-1-yl)pyrimidine (16)

Compound **16** was prepared in a manner similar to that described for **9** in 80% yield as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.94–3.00 (4H, m), 3.83–3.90 (4H, m), 6.83–7.03 (3H, m), 8.10–8.21 (1H, m), 8.37 (1H, d, J = 4.9 Hz). MS (ESI): m/z 277 $[M+H]^+$.

5.1.12. 4-(2,3-Difluorophenyl)-2-(piperazin-1-yl)pyrimidine dihydrochloride (17)

To a stirred solution of *tert*-butyl 4-[4-(2,3-difluorophenyl)pyrimidin-2-yl]piperazine-1-carboxylate (11.3 g, 30.0 mmol) in EtOAc (70 mL) and MeOH (45 mL) was added 4 M HCl in EtOAc (38 mL) dropwise at room temperature. After stirring at room temperature overnight, the mixture was concentrated in vacuo. To the residue was added EtOAc (300 mL) and MeOH (60 mL), and the mixture was stirred at room temperature for 2 h. The solid was collected by filtration and washed with EtOAc to give **17** (9.90 g, 94%) as a pale yellow powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.19 (4H, br s), 4.04 (4H, t, J = 4.0 Hz), 7.15–7.17 (1H, m), 7.35–7.41 (1H, m), 7.58–7.65 (1H, m), 7.83–7.86 (1H, m), 8.57 (1H, d, J = 4.0 Hz), 9.21 (2H, br s). MS (ESI): m/z 277 $[M+H]^+$.

5.1.13. 4-(2,5-Difluorophenyl)-2-(piperazin-1-yl)pyrimidine dihydrochloride (18)

Compound **18** was prepared in a manner similar to that described for **17** in 95% yield as a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.19 (4H, br s), 4.05 (4H, t, J = 4.0 Hz), 7.17–7.19 (1H, m), 7.43–7.47 (2H, m), 7.87–7.92 (1H, m), 8.57 (1H, d, J = 4.0 Hz), 9.29 (2H, br s). MS (ESI): m/z 277 $[M+H]^+$.

5.1.14. 4-(3,4-Difluorophenyl)-2-(piperazin-1-yl)pyrimidine dihydrochloride (19)

To a stirred solution of *tert*-butyl 4-[4-(3,4-difluorophenyl) pyrimidin-2-yl]piperazine-1-carboxylate in EtOAc (70 mL) and MeOH (100 mL) was added 4 M HCl in EtOAc (42 mL) dropwise at room temperature. After stirring at room temperature overnight, the mixture was concentrated in vacuo. To the residue was added EtOAc (300 mL) and MeOH (60 mL), and the mixture was stirred at room temperature for 2 h. The solid was collected by filtration and washed with EtOAc to give **19** (11.7 g, quant.) as a pale yellow powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.19 (4H, br s), 4.07 (4H, t, J = 5.1 Hz), 7.42–7.47 (2H, m), 7.91–7.94 (2H, m), 8.57 (1H, d, J = 5.4 Hz), 9.18 (2H, br s). MS (ESI): m/z 277 $[M+H]^+$.

5.1.15. 4-(3,5-Difluorophenyl)-2-(piperazin-1-yl)pyrimidine dihydrochloride (20)

Compound **20** was prepared in a manner similar to that described for **19** in 95% yield as a yellow powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.19 (4H, br s), 4.07 (4H, t, J = 4.9 Hz), 7.15 (1H, dd, J = 2.4, 5.1 Hz), 7.35–7.40 (1H, m), 7.57–7.59 (1H, m), 8.05–8.09 (1H, dt, J = 1.7, 7.8 Hz), 8.55 (1H, d, J = 5.1 Hz), 9.39 (2H, br s).

5.1.16. 4-[4-(2,4-Difluorophenyl)pyrimidin-2-yl]-*N*-(pyridazin-3-yl)piperazine-1-carboxamide (21a)

Compound **21a** was prepared in a manner similar to that described for **5** in 22% yield as colorless crystals, mp 184–185 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.60–3.68 (4H, m), 3.82–3.91 (4H, m), 7.06 (1H, dd, J = 2.5, 5.1 Hz), 7.23–7.32 (1H, m), 7.38–7.47 (1H, m), 7.58 (1H, dd, J = 5.1, 9.1 Hz), 8.02 (1H, dd, J = 1.5, 9.1 Hz), 8.11–7.21 (1H, m), 8.50 (1H, d, J = 5.1 Hz), 8.85 (1H, dd, J = 1.5, 4.5 Hz), 9.97 (1H, s). MS (ESI): m/z 398 $[M+H]^+$ Anal. Calcd for $C_{19}H_{17}F_2N_7O{\cdot}0.5H_2O$: C, 56.15; H, 4.46; N, 24.13. Found: C, 55.91; H, 4.43; N, 23.93.

5.1.17. 4-[4-(2,3-Difluorophenyl)pyrimidin-2-yl]-*N*-(pyridazin-3-yl)piperazine-1-carboxamide (21b)

A mixture of 4-(2,3-difluorophenyl)-2-(piperazin-1-yl)pyrimidine dihydrochloride (200 mg, 0.573 mmol), phenyl pyridazin-3-ylcarbamate (136 mg, 0.630 mmol), triethylamine (0.319 mL, 2.29 mmol), and acetone (1.0 mL) was stirred at 45 °C for 3 h. The mixture was diluted with water, and stirred at room temperature for 1 h. The solid was collected by filtration, washed with water, and recrystallized from THF–hexane to give **21b** (206 mg, 91%) as colorless crystals, mp 248–249 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.54–3.72 (4H, m), 3.77–3.98 (4H, m), 7.09 (1H, dd, J = 2.5, 5.0 Hz), 7.32–7.43 (1H, m), 7.52–7.67 (2H, m), 7.79–7.91 (1H, m), 8.02 (1H, dd, J = 1.3, 9.0 Hz), 8.54 (1H, d, J = 5.0 Hz), 8.85 (1H, dd, J = 1.3, 4.5 Hz), 9.97 (1H, s). MS (ESI): m/z 398 $[M+H]^+$ Anal. Calcd for $C_{19}H_{17}F_2N_7O$: C, 57.43; H, 4.31; N, 24.67. Found: C, 57.18; H, 4.45; N, 24.57.

5.1.18. 4-[4-(2,5-Difluorophenyl)pyrimidin-2-yl]-*N*-(pyridazin-3-yl)piperazine-1-carboxamide (21c)

Compound **21c** was prepared in a manner similar to that described for **21b** in 44% yield as colorless crystals, mp 271–272 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.55–3.72 (4H, m), 3.79–3.96 (4H, m), 7.11 (1H, dd, J = 2.4, 5.1 Hz), 7.38–7.50 (2H, m), 7.58 (1H, dd, J = 4.7, 9.0 Hz), 7.82–7.93 (1H, m), 8.02 (1H, dd, J = 1.3, 9.0 Hz), 8.53 (1H, d, J = 5.1 Hz), 8.85 (1H, dd, J = 1.3, 4.7 Hz), 9.97 (1H, s). MS (ESI): m/z 398 $[M+H]^+$ Anal. Calcd for $C_{19}H_{17}F_2N_7O$: C, 57.43; H, 4.31; N, 24.67. Found: C, 57.19; H, 4.41; N, 24.54.

5.1.19. 4-[4-(3,4-Difluorophenyl)pyrimidin-2-yl]-*N*-(pyridazin-3-yl)piperazine-1-carboxamide (21d)

Compound **21d** was prepared in a manner similar to that described for **21b** in 83% yield as colorless crystals, mp 218–219 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.56–3.73 (4H, m), 3.80–3.99 (4H, m), 7.30 (1H, d, J = 5.3 Hz), 7.51–7.66 (2H, m), 7.96–8.11 (2H, m), 8.18–8.28 (1H, m), 8.50 (1H, d, J = 5.3 Hz), 8.85 (1H, d, J = 3.4 Hz), 9.97 (1H, s). MS (ESI): m/z 398 $[M+H]^+$ Anal. Calcd for $C_{19}H_{17}F_2N_7O$: C, 57.43; H, 4.31; N, 24.67. Found: C, 57.40; H, 4.46; N, 24.69.

5.1.20. 4-[4-(3,5-Difluorophenyl)pyrimidin-2-yl]-*N*-(pyridazin-3-yl)piperazine-1-carboxamide (21e)

Compound **21e** was prepared in a manner similar to that described for **21b** in 39% yield as colorless crystals, mp 270–271 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.53–3.75 (4H, m), 3.78–4.00 (4H, m), 7.35 (1H, d, J = 5.3 Hz), 7.38–7.49 (1H, m), 7.58 (1H, dd, J = 4.5, 9.1 Hz), 7.82–7.95 (2H, m), 8.02 (1H, dd, J = 1.5, 9.1 Hz), 8.54 (1H, d, J = 5.3 Hz), 8.85 (1H, d, J = 3.4 Hz), 9.97 (1H, s). MS (ESI): m/z 398 $[M+H]^+$ Anal. Calcd for $C_{19}H_{17}F_2N_7O$: C, 57.43; H, 4.31; N, 24.67. Found: C, 57.24; H, 4.33; N, 24.39.

5.1.21. 2,2,2-Trichloroethyl pyridazin-3-ylcarbamate (23)

To a stirred solution of pyridazin-3-amine (2.9 g, 30.5 mmol) and pyridine (7.4 mL, 91.5 mmol) in THF (40 mL) and DMA (20 mL) was added 2,2,2-Trichloroethyl chloroformate (6.31 mL, 45.7 mmol) at 0 °C dropwise. The mixture was stirred at 0 °C for 1.0 h, poured into water, and extracted with EtOAc. The organic layer was washed with water, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was recrystallized from EtOAc–hexane to give **23** (3.76 g, 46%) as an off-white crystals ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.88 (2H, s), 7.50–7.55 (1H, m), 8.25–8.28 (1H, m), 8.74 (1H, br s), 8.95–8.97 (1H, m).

5.1.22. Phenyl pyridazin-3-ylcarbamate (24)

To a stirred suspension of pyridazin-3-amine hydrochloride (10.0 g, 76.0 mmol) and pyridine (13.6 mL, 167 mmol) in MeCN (50 mL) was added phenyl chloroformate (11.4 mL, 91.2 mmol) dropwise at 0 °C. The mixture was stirred at 0 °C for 1 h. To the mixture was added water (100 mL) and the mixture was stirred at room temperature for 30 min and 0 °C for 1 h. The solid was collected by filtration and washed with MeCN/water (1:2) (20 mL), water (20 mL) to give **24** (16.3 g, 87%) as an off-white powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.17–7.33 (3H, m), 7.37–7.47 (2H, m), 7.50 (1H, dd, J = 4.7, 9.0 Hz), 8.29 (1H, dd, J = 1.4, 9.0 Hz), 8.91 (1H, br s), 8.95 (1H, dd, J = 1.4, 4.7 Hz).

5.1.23. *tert*-Butyl 4-(2-chloropyridin-4-yl)piperazine-1-carboxylate (29)

A mixture of 4-bromo-2-chloropyridine (3.87 mL, 34.9 mmol), *N*-Boc-piperazine (5.00 g, 26.9 mmol), sodium *tert*-butoxide (3.87 g, 40.3 mmol), 4,5-bis(diphenylphosphino)-9,9-dimethylxanthene(932.2 mg, 1.61 mmol), tris(dibenzylideneacetone)dipalladium (0) (492 mg, 0.537 mmol), and toluene (270 mL) was stirred at 100 °C overnight under N_2 atmosphere. The mixture was diluted with water, and extracted with EtOAc. The organic layer was washed with water, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane–EtOAc) to give **29** (5.62 g, 70%) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.49 (9H, s), 3.30–3.37 (4H, m), 3.52–3.60 (4H, m), 6.57 (1H, dd, J = 2.4, 6.1 Hz), 6.65 (1H, d, J = 2.4 Hz), 8.04 (1H, d, J = 6.1 Hz).

5.1.24. *tert*-Butyl 4-(5-bromopyridin-3-yl)piperazine-1-carboxylate (30)

Compound **30** was prepared in a manner similar to that described for **29** in 79% yield as a colorless viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.49 (9H, s), 3.14–3.23 (4H, m), 3.55–3.63 (4H, m), 7.28–7.32 (1H, m), 8.15 (1H, d, J = 1.7 Hz), 8.21 (1H, d, J = 1.7 Hz).

5.1.25. *tert*-Butyl 4-(6-chloropyrimidin-4-yl)piperazine-1-carboxylate (31)

Compound **31** was prepared in a manner similar to that described for **29** in 72% yield as a yellow powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.49 (9H, s), 3.50–3.57 (4H, m), 3.61–3.70 (4H, m), 6.50 (1H, s), 8.39 (1H, s).

5.1.26. 2-Chloro-4-(2,4-difluorophenyl)pyridine (33)

To a solution of 4-bromo-2-chloropyridine (30.0 g, 156 mmol), (2,4-difluorophenyl)boronic acid (24.6 g, 156 mmol), and sodium carbonate (43.1 g, 312 mmol) in MeOH (195 mL) was added tetrakis(triphenylphosphine)palladium (0) (9.01 g, 7.79 mmol) at room

temperature under N_2 atmosphere, and the mixture was stirred at 50 °C for 17 h. The reaction mixture was cooled to room temperature and the resulting solid was filtered. The filtrate was dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane–EtOAc) to give **33** (31.9 g, 91%) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.95–7.05 (2H, s), 7.37–7.39 (1H, m), 7.43–7.47 (1H, m), 7.49 (1H, br s), 8.47 (1H, d, J = 13.2 Hz).

5.1.27. *tert*-Butyl 4-[6-(2,4-difluorophenyl)pyridin-2-yl]piperazine-1-carboxylate (34)

To a solution of *tert*-butyl 4-(6-bromopyridin-2-yl)piperazine-1-carboxylate (3.00 g, 8.77 mmol) and (2,4-difluorophenyl)boronic acid (1.40 g, 12.3 mmol) in 1,2-dimethoxyethane (30 mL) and water (3.0 mL) was added sodium carbonate (1.25 g, 17.5 mmol) and tetrakis(triphenylphosphine)palladium (0) (340 mg, 0.440 mmol) at room temperature under N_2 atmosphere and heated under reflux for 1 h. The reaction mixture was cooled to room temperature, poured into water, and extracted with EtOAc. The extract was washed with aqueous saturated sodium hydrogen carbonate solution, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane–EtOAc) to give **34** (2.84 g, 86%) as a colorless viscous oil. This product was used for next reaction without further purification.

5.1.28. *tert*-Butyl 4-[2-(2,4-difluorophenyl)pyridin-4-yl]piperazine-1-carboxylate (35)

Compound **35** was prepared in a manner similar to that described for **8** in 37% yield as a yellow viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.49 (9H, s), 3.34–3.41 (4H, m), 3.54–3.63 (4H, m), 6.65 (1H, dd, J = 2.5, 5.9 Hz), 6.83–7.03 (2H, m), 7.08–7.14 (1H, m), 7.87–8.00 (1H, m), 8.39 (1H, d, J = 5.9 Hz).

5.1.29. *tert*-Butyl 4-[5-(2,4-difluorophenyl)pyridin-3-yl]piperazine-1-carboxylate (36)

Compound **36** was prepared in a manner similar to that described for **8** in 94% yield as a yellow viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.49 (9H, s), 3.18–3.26 (4H, m), 3.57–3.65 (4H, m), 6.88–7.04 (2H, m), 7.28–7.32 (1H, m), 7.35–7.46 (1H, m), 8.24 (1H, s), 8.31 (1H, d, J = 2.8 Hz).

5.1.30. *tert*-Butyl 4-[4-(2,4-difluorophenyl)pyridin-2-yl]piperazine-1-carboxylate (37)

A mixture of 2-chloro-4-(2,4-difluorophenyl)pyridine (15.0 g, 66.5 mmol), *N*-Boc-piperazine (12.4 g, 66.6 mmol), palladium acetate (746 mg, 3.32 mmol), 2,2-bis(diphenylphosphino)-1,1-binaphthyl (3.31 g, 5.32 mmol), sodium *tert*-butoxide (13.0 g, 133 mmol), and 1,4-dioxane (133 mL) was stirred at 85 °C for 18 h. The reaction mixture was cooled to room temperature and the solvent was distilled off under reduced pressure. To the residue was added EtOAc, washed with water, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane–EtOAc) to give **37** (19.0 g, 76%) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.49 (9H, s), 3.57 (8H, s), 6.75–6.78 (2H, m), 6.90–6.98 (2H, m), 7.39–7.45 (1H, m), 8.24 (1H, d, J = 4.8 Hz). MS (ESI): m/z 376 $[M+H]^+$.

5.1.31. *tert*-Butyl 4-[6-(2,4-difluorophenyl)pyrimidin-4-yl]piperazine-1-carboxylate (38)

Compound **38** was prepared in a manner similar to that described for **8** in 72% yield as a yellow viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.50 (9H, s), 3.52–3.60 (4H, m), 3.68–3.75 (4H, m), 6.86–7.06 (3H, m), 8.05–8.16 (1H, m), 8.70 (1H, d, J = 1.1 Hz). MS (ESI): m/z 377 $[M+H]^+$

5.1.32. 1-[6-(2,4-Difluorophenyl)pyridin-2-yl]piperazine (39)

A solution of *tert*-butyl 4-[6-(2,4-difluorophenyl)pyridin-2-yl]piperazine-1-carboxylate (2.84 g, 7.55 mmol) in trifluoroacetic acid–methylene chloride (1:2) was stirred at room temperature for 2 h and the mixture was concentrated in vacuo. The residue was neutralized by adding aqueous saturated sodium hydrogen carbonate solution, and extracted with methylene chloride. The extract was dried over anhydrous Na_2SO_4 and the solvent was distilled off under reduced pressure. The residue was recrystallized from hexane and Et_2O to give **39** (1.50 g, 72%) as colorless crystals. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.97–3.05 (4H, m), 3.39–3.41 (4H, m), 6.55 (1H, d, J = 8.4 Hz), 6.79–6.82 (1H, m), 6.86–6.88 (1H, m), 7.08 (1H, dd, J = 2.4, 7.6 Hz), 7.46 (1H, t, J = 8.0 Hz), 7.95 (1H, q, J = 8.8 Hz).

5.1.33. 1-[2-(2,4-Difluorophenyl)pyridin-4-yl]piperazine (40)

Compound **40** was prepared in a manner similar to that described for **9** in 91% yield as a pale yellow viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.94–3.06 (4H, m), 3.26–3.39 (4H, m), 6.65 (1H, dd, J = 2.5, 5.9 Hz), 6.81–7.02 (2H, m), 7.11 (1H, t, J = 2.0 Hz), 7.84–7.99 (1H, m), 8.37 (1H, d, J = 5.9 Hz).

5.1.34. 1-[5-(2,4-Difluorophenyl)pyridin-3-yl]piperazine (41)

Compound **41** was prepared in a manner similar to that described for **9** in 94% yield as a pale yellow viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.99–3.12 (4H, m), 3.16–3.28 (4H, m), 6.87–7.04 (2H, m), 7.24–7.33 (1H, m), 7.35–7.47 (1H, m), 8.21 (1H, s), 8.31 (1H, d, J = 2.8 Hz).

5.1.35. 1-[4-(2,4-Difluorophenyl)pyridin-2-yl]piperazine (42)

To a stirred solution of *tert*-butyl 4-[4-(2,4-difluorophenyl)pyridin-2-yl]piperazine-1-carboxylate (19.0 g, 50.6 mmol) in MeOH (63 mL) was added 4 N HCl in MeOH (50 mL) dropwise at room temperature. After stirring at room temperature for 16 h, the resulting solid was collected by filtration and washed with EtOAc to give **42** (15.5 g, 98%) as an off-white powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.22 (4H, s), 3.91 (4H, s), 7.01–7.02 (1H, m), 7.23 (1H, br s), 7.26–7.31 (1H, m), 7.44–7.50 (1H, m), 7.72–7.78 (1H, m), 8.20 (1H, d, J = 5.6 Hz), 9.40 (2H, br s). MS (ESI): m/z 276 $[M+H]^+$.

5.1.36. 4-(2,4-Difluorophenyl)-6-(piperazin-1-yl)pyrimidine (43)

Compound **43** was prepared in a manner similar to that described for **9** in 83% yield as a pale yellow viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.91–3.03 (4H, m), 3.63–3.74 (4H, m), 6.83–7.06 (3H, m), 8.01–8.15 (1H, m), 8.68 (1H, s). MS (ESI): m/z 277 $[M+H]^+$.

5.1.37. 4-[6-(2,4-Difluorophenyl)pyridin-2-yl]-*N*-(pyridazin-3-yl)piperazine-1-carboxamide (44a)

Compound **44a** was prepared in a manner similar to that described for **5** in 52% yield as colorless crystals, mp 200–201 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.62–3.64 (8H, m), 6.90 (1H, d, J = 9.2 Hz), 7.07 (1H, dd, J = 2.4, 4.8 Hz), 7.18–7.22 (1H, m), 7.30–7.36 (1H, m), 7.56 (1H, dd, J = 4.4, 6.8 Hz), 7.65 (1H, t, J = 8.0 Hz), 7.97–8.05 (2H, m), 8.83 (1H, dd, J = 1.2, 3.0 Hz), 9.94 (1H, br s). MS (ESI): m/z 397 $[M+H]^+$ analytical HPLC showed 100% purity.

5.1.38. 4-[2-(2,4-Difluorophenyl)pyridin-4-yl]-*N*-(pyridazin-3-yl)piperazine-1-carboxamide (44b)

Compound **44b** was prepared in a manner similar to that described for **5** in 57% yield as colorless crystals, mp 189–190 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.40–3.51 (4H, m), 3.62–3.72 (4H, m), 6.90 (1H, dd, J = 2.5, 5.9 Hz), 7.12–7.25 (2H, m), 7.29–7.40 (1H, m), 7.58 (1H, dd, J = 4.6, 9.1 Hz), 7.82–8.05 (2H, m),

8.30 (1H, d, J = 5.9 Hz), 8.83–8.88 (1H, m), 9.99 (1H, s). MS (ESI): m/z 397 $[M+H]^+$ analytical HPLC showed 95.8% purity.

5.1.39. 4-[5-(2,4-Difluorophenyl)pyridin-3-yl]-*N*-(pyridazin-3-yl)piperazine-1-carboxamide (44c)

Compound **44c** was prepared in a manner similar to that described for **5** in 22% yield as colorless crystals, mp 172–173 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.23–3.42 (4H, m), 3.60–3.76 (4H, m), 7.17–7.29 (1H, m), 7.35–7.51 (2H, m), 7.54–7.74 (2H, m), 7.97–8.07 (1H, m), 8.16 (1H, s), 8.38 (1H, d, J = 2.6 Hz), 8.80–8.90 (1H, m), 10.01 (1H, s). MS (ESI): m/z 397 $[M+H]^+$ Anal. Calcd for $C_{20}H_{18}F_2N_6O\cdot0.5H_2O$: C, 59.25; H, 4.72; N, 20.73. Found: C, 59.36; H, 4.68; N, 20.55.

5.1.40. 4-[4-(2,4-Difluorophenyl)pyridin-2-yl]-*N*-(pyridazin-3-yl)piperazine-1-carboxamide (44d)

Compound **44d** was prepared in a manner similar to that described for **5** in 31% yield as a colorless crystals, mp 188–189 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.63 (8H, s), 6.82–6.84 (1H, m), 6.98 (1H, s), 7.21–7.26 (1H, m), 7.38–7.44 (1H, m), 7.56–7.60 (1H, m), 7.66–7.72 (1H, m), 8.00–8.03 (1H, m), 8.21 (1H, d, J = 5.2 Hz), 8.85 (1 H, d, J = 3.6 Hz), 9.96 (1H, s). MS (ESI): m/z 397 $[M+H]^+$ analytical HPLC showed 98.3% purity.

5.1.41. 4-[6-(2,4-Difluorophenyl)pyrimidin-4-yl]-*N*-(pyridazin-3-yl)piperazine-1-carboxamide (44e)

Compound **44e** was prepared in a manner similar to that described for **5** in 10% yield as colorless crystals, mp 249–250 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.60–3.69 (4H, m), 3.71–3.80 (4H, m), 7.16 (1H, s), 7.20–7.29 (1H, m), 7.36–7.47 (1H, m), 7.59 (1H, dd, J = 4.6, 9.1 Hz), 7.94–8.05 (2H, m), 8.63 (1H, d, J = 1.4 Hz), 8.85 (1H, dd, J = 1.4, 4.6 Hz), 9.98 (1H, s). MS (ESI): m/z 398 $[M+H]^+$ Anal. Calcd for $C_{19}H_{17}F_2N_7O\cdot0.5H_2O$: C, 56.15; H, 4.46; N, 24.13. Found: C, 55.86; H, 4.24; N, 24.17.

5.2. Solubility determination

Small volumes of the compound DMSO solutions were added to the aqueous buffer solution (pH 6.8). After incubation, precipitates were separated by filtration. The solubility was determined by HPLC analysis of each filtrate.

5.3. Measurement of FAAH inhibitory activity

5.3.1. Preparation of enzyme fraction

The FAAH gene was cloned by PCR. That is, an amplified fragment was obtained by carrying out the reaction at 95 °C. For 30 s and at 55 °C. for 30 sec in one cycle and at 72 °C. for 2 min in 45 cycles, using a human brain library as cDNA library, and using 5′-AAAAGAATTCGCCACCATGGTGCAGTACGAGCTGTG-3′[SEQ ID NO:1] and 5′-TTTTGTCGACTCAGGATGACTGCTTTT-3′ [SEQ ID NO:2] as primer set, and KOD DNA polymerase (Toyobo Co., Ltd) as a DNA polymerase. The amplified fragment was cleaved with restriction enzymes EcoRI and SalI, recovered, and then was inserted into a pMSRαvector which had been cleaved with the same restriction enzymes EcoRI and SalI and recovered, thereby to obtain pMSRα-human FAAH. A cell line CHO-K1/human was prepared, in which human FAAH was stably expressed in the cell line CHO-K1 by a method known perse, using the above-obtained plasmid. The CHO-K1/human FAAH was cultured in a CO_2 incubator at 37 °C, using a medium in which fetal bovine serum (FBS) and G418 were added to Ham's F-12 medium to final concentrations of 10% and 800 μg/mL, respectively, and then the cells were harvested. After washing with PBS, the cells were suspended in a buffer (10 mM Tris, 1 mM EDTA and 10 mM $MgCl_2$, all at final concentrations) and disrupted with a Polytron homogenizer. After centrifugation at 900g, the supernatant was recovered and further centrifuged at 10,000g. A pellet obtained therefrom was suspended in M-PER (Catalog No. 78501; Pierce Biotechnology, Inc.) to give an enzyme fraction.

5.3.2. Enzyme reaction

A test compound of various concentration, the enzymatic fraction (final concentration of 125–250 ng) and AMC substrate arachidonoyl amide (AMCAA: manufactured by CAYMAN CHEMICAL: final concentration of 3 μM) were reacted in reaction buffer (Tris–HCl (pH 9.0) of 125 mM, EDTA of 1 mM, HEPES of 0.4 mM, glycerol of 0.2%, Triton X-100 of 0.02% and fatty acid free BSA of 0.3% as final concentrations) at 37 °C for 90 min. After the reaction, the fluorescent count of the plate was measured by a ARVOSX 1420 MULTILABEL COUNTER (manufactured by WALLAC) or EnVision (Perkinelmer) under excitation at 355 nm and emission at 460 nm. The count of a sample containing solvent instead of the test compound was taken as 100%, and the count at zero time was taken as 0%, to calculate the inhibitory activity of the compound.

5.4. Evaluation of analgesic effect in mouse acetic acid-induced writhing test

Male ICR mice (25–40 g) purchased from CLEA Japan Inc. (Tokyo, Japan) were used in experiments. All of the animals were housed in cages with free access to food and water. Experiments were conducted between 9:00 and 17:00 h to minimize the diurnal variation. The care and use of animals and the experimental protocols used in this study were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company Limited. Compounds were suspended to 0.5% methylcellulose solution (Wako Pure Chemical Industries Limited, Osaka, Japan) and orally administered to mice at a volume of 10 mL/kg. After 60 min of drug administration, 0.6% (v/v) acetic acid (Wako Pure Chemical Industries Limited, 10 mL/kg) was intraperitoneally injected and each mouse was then placed in an individual clear plastic observation chamber. The number of stretching movements (arching of back, development of tension in the abdominal muscles, elongation of the body, and extention of the forelimbs) made by each mouse was counted for 20 min.

5.5. Analgesic effect of compound 21d in rat neuropathic pain model

Seven-week-old male Sprague Dawley rats were used in this experiment. To prepare spared nerve injured rats, the right common peroneal nerve was exposed and tightly ligated with 4-0 silk sutures followed by transection at the distal side under anesthetization with pentobarbital. To prepare sham-operated rats, the right common peroneal nerve was exposed, but left uninjured. The rats were orally administered with compound, **21d** at 1, 3, 10 mg/kg or 0.5% methylcellulose as vehicle 7 days after SNI operation. Then tactile allodynia of ipsilateral hind paw was assessed by von Frey filaments in a blinded manner 2 h after drug treatment. Fifty percent threshold (g) of paw withdrawal response to tactile stimulation was determined using the up-down method with starting the filament of 4.56 g.

5.6. Analgesic effect of compound 21d in rat inflammatory pain model

Six-week-old male Sprague Dawley rats were used in this experiment. To prepare CFA-treated rats, 150 μL of CFA was injected into the plantar surface of the right hind paw. To prepare sham-operated rats, liquid paraffin, vehicle, was injected instead

of CFA. The rats were orally administered with compound, **21d** at 1, 3, 10 mg/kg or 0.5% methylcellulose as vehicle 5 days after CFA injection. Then tactile allodynia of ipsilateral hind paw was assessed by von Frey filaments in a blinded manner 4 h after drug treatment. Fifty percent threshold (g) of paw withdrawal response to tactile stimulation was determined using the up-down method with starting the filament of 4.56 g.

Acknowledgments

We thank Mr. Takashi Santou for the excellent technical assistance for conducting biological assays; Dr. Satoshi Yamamoto for helpful discussions.

References and notes

1. Cravatt, B. F.; Giang, D. K.; Mayfield, S. P.; Boger, D. L.; Lerner, R. A.; Gilula, N. B. *Nature* **1996**, *384*, 83.
2. Giang, D. K.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2238.
3. Deutsch, D. G.; Ueda, N.; Yamamoto, N. *Prostaglandins Leukot. Essent. Fatty Acids* **2002**, *66*, 201.
4. Patricelli, M. P.; Cravatt, B. F. *Vitam. Horm.* **2001**, *62*, 95.
5. Egertova, M.; Cravatt, B. F.; Elphick, M. R. *Neuroscience* **2003**, *119*, 481.
6. Boger, D. L.; Fecik, R. A.; Patterson, J. E.; Miyauchi, H.; Patricelli, M. P.; Cravatt, B. F. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2613.
7. Ezzili, C.; Otrubova, K.; Boger, D. L. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 5959.
8. Devane, W. A.; Hanus, L.; Breuer, A.; Pertwee, R. G.; Stevenson, L. A.; Griffin, G.; Gibson, D.; Mandelbaum, A.; Etinger, A.; Mechoulam, R. *Science* **1992**, *1946*, 258.
9. Martin, B. R.; Mechoulam, R.; Razdan, R. K. *Life Sci.* **1999**, *65*, 573.
10. Di Marzo, V.; Bisogno, T.; De Petrocellis, L.; Melck, D.; Martin, B. R. *Curr. Med. Chem.* **1999**, *6*, 721.
11. Schmid, H. H. O.; Schmid, P. C.; Natarajan, V. *Prog. Lipid Res.* **1990**, *29*, 1.
12. Boger, D. L.; Henriksen, S. J.; Cravatt, B. F. *Curr. Pharm. Des.* **1998**, *4*, 303.
13. Cravatt, B. F.; Lerner, R. A.; Boger, D. L. *J. Am. Chem. Soc.* **1996**, *118*, 580.
14. Cravatt, B. F.; Prospero-Garcia, O.; Suizdak, G.; Gilula, N. B.; Henriksen, S. J.; Boger, D. L.; Lerner, R. A. *Science* **1995**, *268*, 1506.
15. Lerner, R. A.; Siuzdak, G.; Prospero-Garcia, O.; Henriksen, S. J.; Boger, D. L.; Cravatt, B. F. *Proc. Natl. Sci. U.S.A.* **1994**, *91*, 9505.
16. Rodriguez de Fonseca, F.; Navarro, M.; Gomez, R.; Escuredo, L.; Nava, F.; Fu, J.; Murillo-Rodriguez, E.; Giuffrida, A.; Lo Verme, J.; Gaetani, S.; Kathuria, S.; Gall, C.; Piomelli, D. *Nature* **2001**, *414*, 219.
17. Goparaju, S. K.; Ueda, N.; Taniguchi, K.; Yamamoto, S. *Biochem. Pharmacol.* **1999**, *57*, 417.
18. Walker, J. M.; Huang, S. M.; Strangman, N. M.; Tsou, K.; Sanudo-Pena, M. C. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 12198.
19. Lambert, D. M.; Fowler, C. J. *J. Med. Chem.* **2005**, *48*, 5059.
20. Pacher, P.; Batkai, S.; Kunos, G. *Pharmacol. Rev.* **2006**, *58*, 389.
21. Di Marzo, V. *Nat. Rev. Drug Disc.* **2008**, *7*, 438.
22. Thabuis, C.; Destaillats, F.; Tissot-Favre, D.; Martin, J. C. *Lipid Technol.* **2007**, *19*, 225.
23. Cravatt, B. F.; Demarest, K.; Patricelli, M. P.; Bracey, M. H.; Giang, D. K.; Martin, B. R.; Lichtman, A. H. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 9371.
24. Angela, B.; Clement, E.; Gregory, H.; Aron, H. L.; Benjamin, F. C. *J. Neurosci.* **2003**, *23*, 3916.
25. Lichtman, A. H.; Shelton, C. C.; Advani, T.; Cravatt, B. F. *Pain* **2004**, *109*, 319.
26. Matsuda, L. A.; Lolait, S. J.; Brownstein, M. J.; Young, A. C.; Bonner, T. I. *Nature* **1990**, *346*, 561.
27. Richardson, J. D.; Aanonsen, L.; Hargreaves, K. M. *Eur. J. Pharmacol.* **1998**, *345*, 145.
28. Di Marzo, V.; De Petrocellis, L.; Fezza, F.; Ligresti, A.; Bisogno, T. *Prostaglandins Leukot. Essent. Fatty Acids* **2002**, *66*, 377.
29. Patel, K. D.; Davison, J. S.; Pittman, Q. J.; Sharkey, K. A. *Curr. Med. Chem.* **2010**, *14*, 1393.
30. Benito, C.; Tolón, R. M.; Pazos, M. R.; Núñez, E.; Castillo, A. L.; Romero, J. *Br. J. Pharmacol.* **2008**, *2*, 277.
31. Moreira, F. A.; Grieb, M.; Lutz, B. *Best Pract. Res., Clin. Endocrinol. Metab.* **2009**, *12*, 1647.
32. Karst, M.; Wippermann, S. *Expert Opin. Invest. Drugs* **2009**, *2*, 125.
33. Johnson, D. S.; Stiff, C.; Lazerwith, S. E.; Kesten, S. R.; Fay, L. K.; Morris, M.; Beidler, D.; Liimatta, M. B.; Smith, S. E.; Dudley, D. T.; Sadagopan, N.; Bhattachar, S. N.; Kesten, S. J.; Nomanbhoy, T. K.; Cravatt, B. F.; Ahn, K. *ACS Med. Chem. Lett.* **2011**, *2*, 91.
34. Keith, J. M.; Apodaca, R.; Tichenor, M.; Xiao, W.; Jones, W.; Pierce, J.; Seierstad, M.; Palmer, J.; Webb, M.; Karbarz, M.; Scott, B.; Wilson, S.; Luo, L.; Wennerholm, M.; Chang, L.; Brown, S.; Rizzolio, M.; Rynberg, R.; Chaplan, S.; Breitenbucher, J. G. *ACS Med. Chem. Lett.* **2012**, *3*, 823.
35. Kathuria, S.; Gaetani, S.; Fegley, D.; Valino, F.; Duranti, A.; Tontini, A.; Mor, M.; Tarzia, G.; Rana, G. L.; Calignano, A.; Giustino, A.; Tattoli, M.; Palmery, M.; Cuomo, V.; Piomelli, D. *Nat. Med.* **2003**, *9*, 76.
36. Abouab-Dellah, A.; Burnier, P.; Hoornaert, C.; Jeunesse, J.; Puech, F. Patent WO 2004/099176, 2004; US 2006/0089344, 2006.
37. Boger, D. L.; Miyauchi, H.; Du, W.; Hardouin, C.; Fecik, R. A.; Cheng, H.; Hwang, I.; Hedrick, M. P.; Leung, D.; Acevedo, O.; Guimaraes, C. R. W.; Jorgensen, W. L.; Cravatt, B. F. *J. Med. Chem.* **2005**, *1849*, 48.
38. Kono, M.; Matsumoto, T.; Kawamura, T.; Nishimura, A.; Kiyota, Y.; Oki, H.; Miyazaki, J.; Igaki, S.; Craig, A. B.; Shimojo, M.; Kori, M. *Bioorg. Med. Chem.* **2013**, *21*, 28.
39. Our FAAH inhibitors are disclosed in our patent. Kori, M.; Kouno, M. Patent WO 2009/048101.
40. Chiang, Y. P.; Novomisle, W. A.; Welch, W. M. Patent WO 2003/000663.
41. Thomas, B. W.; David, B. R. J. *Tetrahedron Lett.* **1967**, *27*, 2555.
42. Alexander, J. P.; Cravatt, B. F. *Chem. Biol.* **2005**, *12*, 1179.
43. Mileni, M.; Garfunkle, J.; DeMartino, J. K.; Cravatt, B. F.; Boger, D. L.; Stevens, R. C. *J. Am. Chem. Soc.* **2009**, *30*, 10497.
44. Mileni, M.; Garfunkle, J.; Ezzili, C.; Kimball, F. S.; Cravatt, B. F.; Stevens, R. C.; Boger, D. L. *J. Med. Chem.* **2010**, *1*, 230.