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Synthesis, SAR study, and biological evaluation of a series of piperazine ureas as fatty acid amide hydrolase (FAAH) inhibitors

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ABSTRACT

A series of piperazine ureas was designed, synthesized, and evaluated for their potential as novel orally available fatty acid amide hydrolase (FAAH) inhibitors that are therapeutically effective against pain. We carried out an optimization study of the lead compound **3** to improve its DMPK profile as well as in vitro potency. We identified the thiazole compound **60j** with potent inhibitory activity, high brain permeability, and good bioavailability. Compound **60j** showed a potent and dose-dependent anti-nociceptive effect in the acetic acid-induced writhing test in mice.

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1. Introduction

Fatty acid amide hydrolase (FAAH)[1–3] is an intracellular serine hydrolase that catalyzes the deactivating hydrolysis of several endogenous lipid amides[4–7] such as endogenous cannabinoid (EC), anandamide (arachidonoylethanolamide: AEA),[8–11] oleamide,[12–15] oleoylethanolamide,[16] and palmitoylethanolamide.[17] These lipid amides have several physiological effects, including the alleviation of pain,[18–21] promotion of sleep,[12,14,15] and regulation of appetite.[22] FAAH knockout mice are viable and healthy, and showed an analgesic phenotype in several neuropathic and inflammatory pain models associated with increased brain AEA levels.[23–25]

AEA is an endogenous cannabinoid that activates the cannabinoid 1 (CB1)/cannabinoid 2 (CB2) receptors. The CB1 receptor is highly expressed in the hippocampus, striatum, nigra, olfactory bulb, and cerebellum in the central nervous system, and suppressively controls the release of neurotransmitters from sensory nerves.[26–28] On the other hand, the CB2 receptor is highly expressed in immune organs such as the spleen, and is implicated in the regulation of inflammatory and immune responses.[29,30] A therapeutic approach utilizing the activation of the CB1 receptor is expected to be beneficial for the treatment of pain, anxiety, depression, and sleep disorders. Although CB1 agonists have potent pharmacological effects, there is concern about adverse effects arising from their systemic activation, such as sedation, dependence, cognitive impairment, psychosis, and affection of the cardiovascular system.[31,32] FAAH inhibitors enhance the activation of CB receptors by blocking the degradation of AEA, but they may offer site-specific increase of AEA in tissues where ECs are being produced by physiological protective mechanisms, suggesting that they exhibit pharmacological effects with less adverse effects. Moreover, FAAH inhibitors may have anti-inflammatory effects by suppressing the release of inflammatory chemical mediators by stimulating the CB2 receptor in immune cells.

To date, several groups have reported various classes of FAAH inhibitors including carbamates (e.g., URB597[33] and SA-47[34]), urea derivatives (e.g., PF-750[35] and LY2183240[36]), and keto-heterocycles (e.g., OL-135[37]) (Fig. 1). Carbamates and urea-type FAAH inhibitors are regarded as irreversible inhibitors that form covalent tight

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; Troc, 2,2,2-trichloroethoxy carbonyl.

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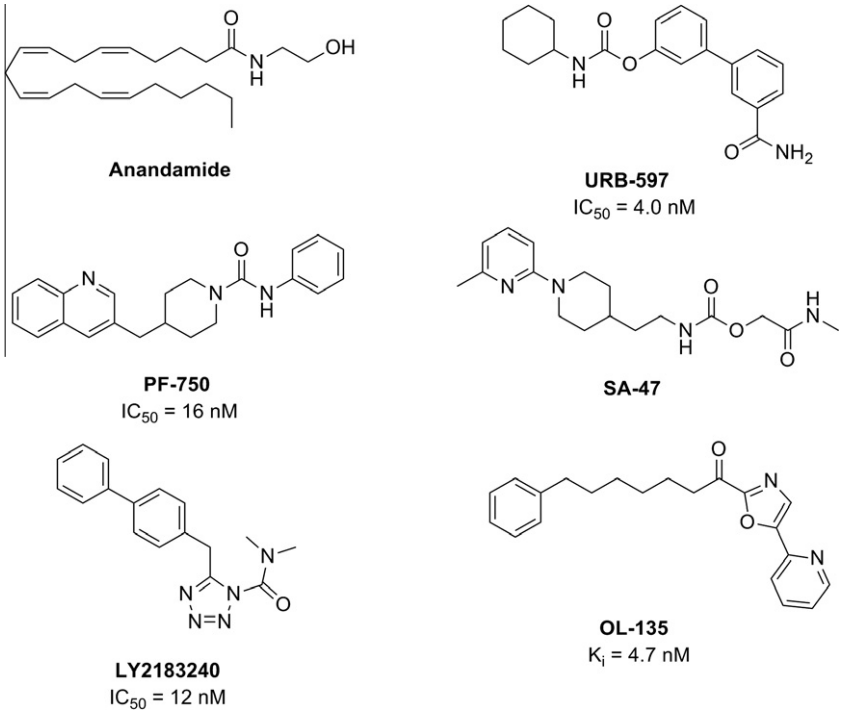


Figure 1. Anandamide (AEA) and known FAAH inhibitors.

binding with the catalytic Ser241 residue within the active site of the FAAH enzyme.[38] On the other hand, reversible inhibitors such as potent keto-heterocycles are believed to form a hemiacetal bond with the catalytic Ser residue.[39,40]

In the course of our exploration of novel FAAH inhibitors, high-throughput screening of our chemical library identified piperazine–urea **1**[41] and aryl carbamate **2**, and we started to design novel FAAH inhibitors from these compounds.[42] Initially, our design was based on the hypothesis that the benzoisoxazolyl carbamate structure of compound **2** (region **b**) corresponded to the phenyl urea moiety (region **a**) of compound **1**. If the benzoisoxazolyl group contributed to the FAAH inhibitory activity of compound **2**, it was thought that the replacement of the benzene ring in region **a** of **1** with a benzoisoxazolyl group would enhance the inhibitory activity. On the basis of this hypothesis, we synthesized a hybrid compound **3** possessing region **c**, and found that **3** showed more potent inhibition than compounds **1** and **2** (Fig. 2). However, compound **3** had poor aqueous solubility (0.09 μg/mL in pH 6.8 solution) and bioavailability (F = 5.0% in rats). Therefore, we performed an optimization study based on this compound as a lead to improve the DMPK profile as well as the in vitro potency. First, we established the putative binding model between the lead compound **3** and human FAAH using the X-ray crystal structure of rat FAAH protein[43] (Fig. 3). This model suggested that the urea moiety was essential for in vitro activity by interacting with the catalytic residues Ser241 and Ser217. The terminal benzene ring was assumed to make a hydrophobic interaction with the lipophilic site. Thr236 and Thr488 are located near the benzoisoxazole and thiadiazole moieties, respectively, indicating that these residues can interact with heteroatoms in the heterocycles of the ligand. On the basis of this modeling information, we investigated the modification of compound **3** for the thiadiazole (**X**), central piperazine (**Y**), terminal heterocycle (**Z**), and substituents on the other terminal benzene ring (**R**). In this paper, we describe the synthesis, biological evaluation, and structure–activity relationship (SAR) study of a series of piperazine ureas as novel FAAH inhibitors.

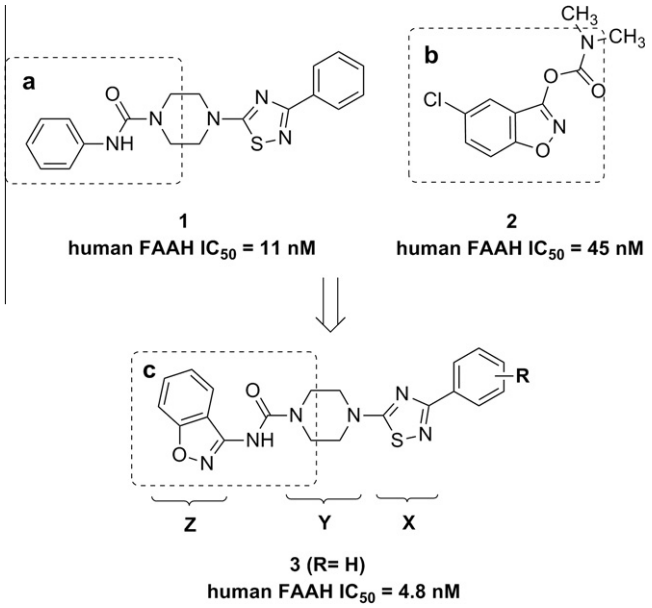


Figure 2. Thiadiazolylpiperazine urea derivative as a promising lead compound.

2. Chemistry

The general synthetic method for the key intermediates **8**–**11** and **23**–**28**, halogenated 5-membered heterocycles, is shown in Scheme 1. The aryl amidine hydrochlorides **4**–**7** were treated with perchloromethyl mercaptan under strongly basic conditions to afford the thiadiazole intermediates **8**–**11**.[44] Thiocyanation of the 2-bromo-acetophenone derivatives **12**–**16** was carried out to give **17**–**21**, and subsequent cyclization under acidic conditions furnished the thiazole intermediates **23**–**27**.[45] The thiophene intermediate **28** was obtained by the bromination of 3-phenylthiophene **22**.

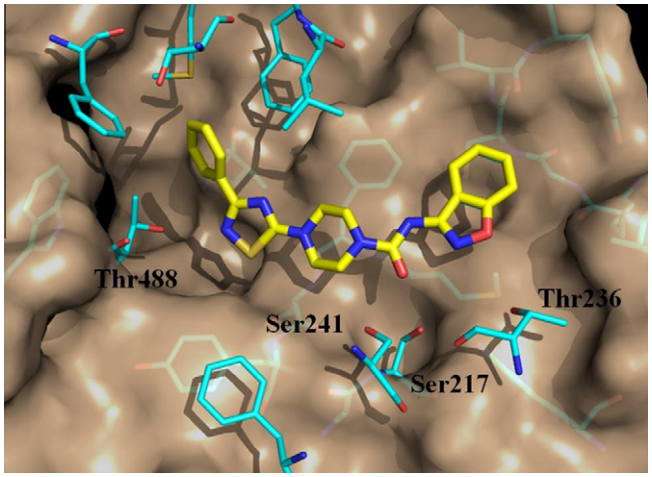


Figure 3. Putative binding model of human FAAH with compound **3**.

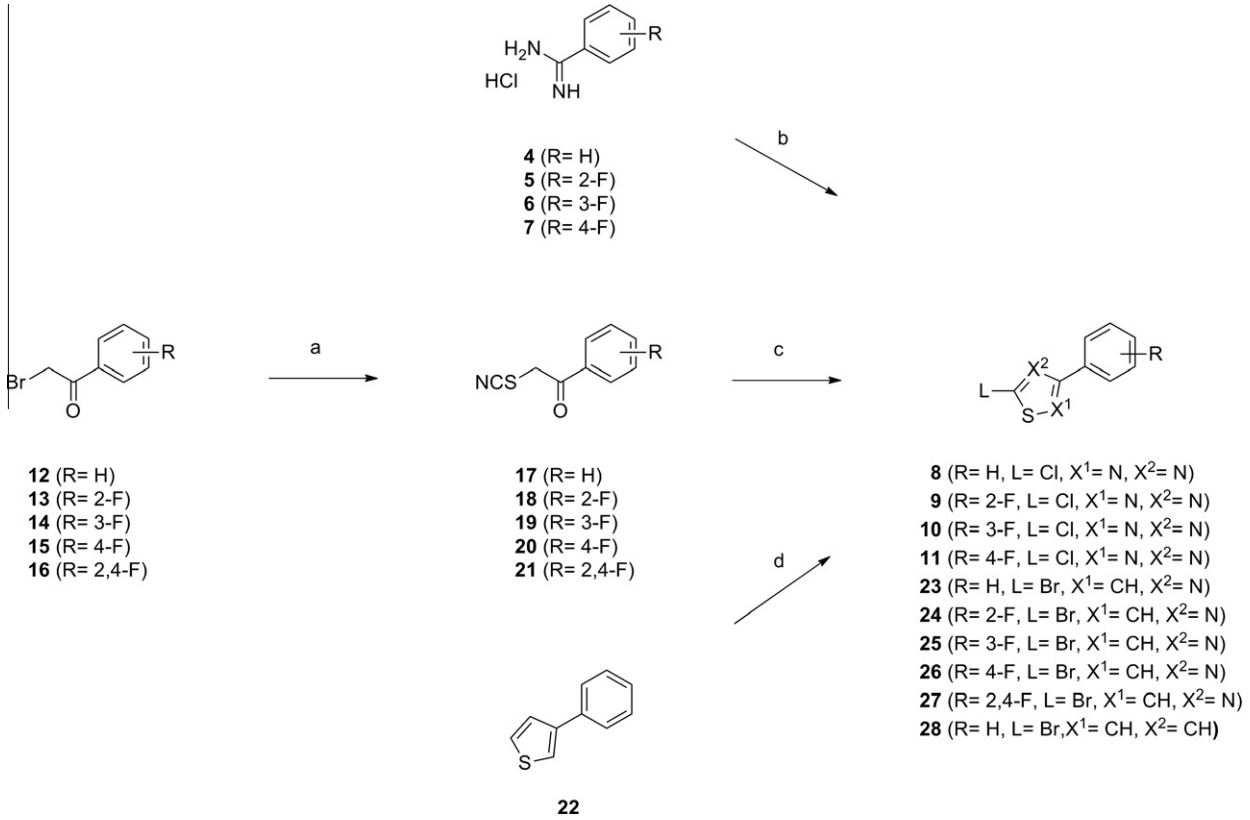
Compounds **43a**–**43d** and **60a**–**60l** were synthesized as shown in Scheme 2. Starting from the heterocyclic amines **29**–**33**, carbamylation with 2,2,2-trichloroethyl chloroformate[46] gave the 2,2,2-trichloroethoxy carbonyl (Troc) derivatives **34**–**38**. Urea formation of the Troc derivatives **34** and **38** with commercially available *N*-Boc-piperazine and subsequent deprotection yielded **41** and **42**. Treatment of compound **41** and **42** with **9**–**11** or 5-chloro-3-phenyl-1,2,4-oxadiazole generated compounds **43a**–**43d**. Compounds **44**–**49** and **51** were synthesized from the corresponding intermediates **8** and **23**–**28** by the coupling reaction in the presence of base or by Buchwald amination with the *N*-protected piperazine reagent. Thiazole ring formation of 2-bromoacetophenone **4** with *N*-Boc-4-aminothiocarbonylpiperidine gave compound **50**. Acidic or basic deprotection and subsequent urea formation with the corresponding Troc derivatives **34**–**38** afforded compounds **60a**–**60l**.

The conversion of the central piperazine ring is illustrated in Scheme 3. The coupling reaction of several amines with 5-chloro-3-phenyl-1,2,4-thiadiazole **8**, followed by deprotection afforded compounds **62a**–**62d**. Amidation of **62a** with 3-aminobenzoisoxazole and urea formation of **62b**–**62d** with compound **38** provided compounds **65a**–**65d**. Compound **65e** was obtained by the coupling reaction of **8** with amine **64** derived from urea formation of *N*-Boc-ethylenediamine with compound **38** and subsequent deprotection.

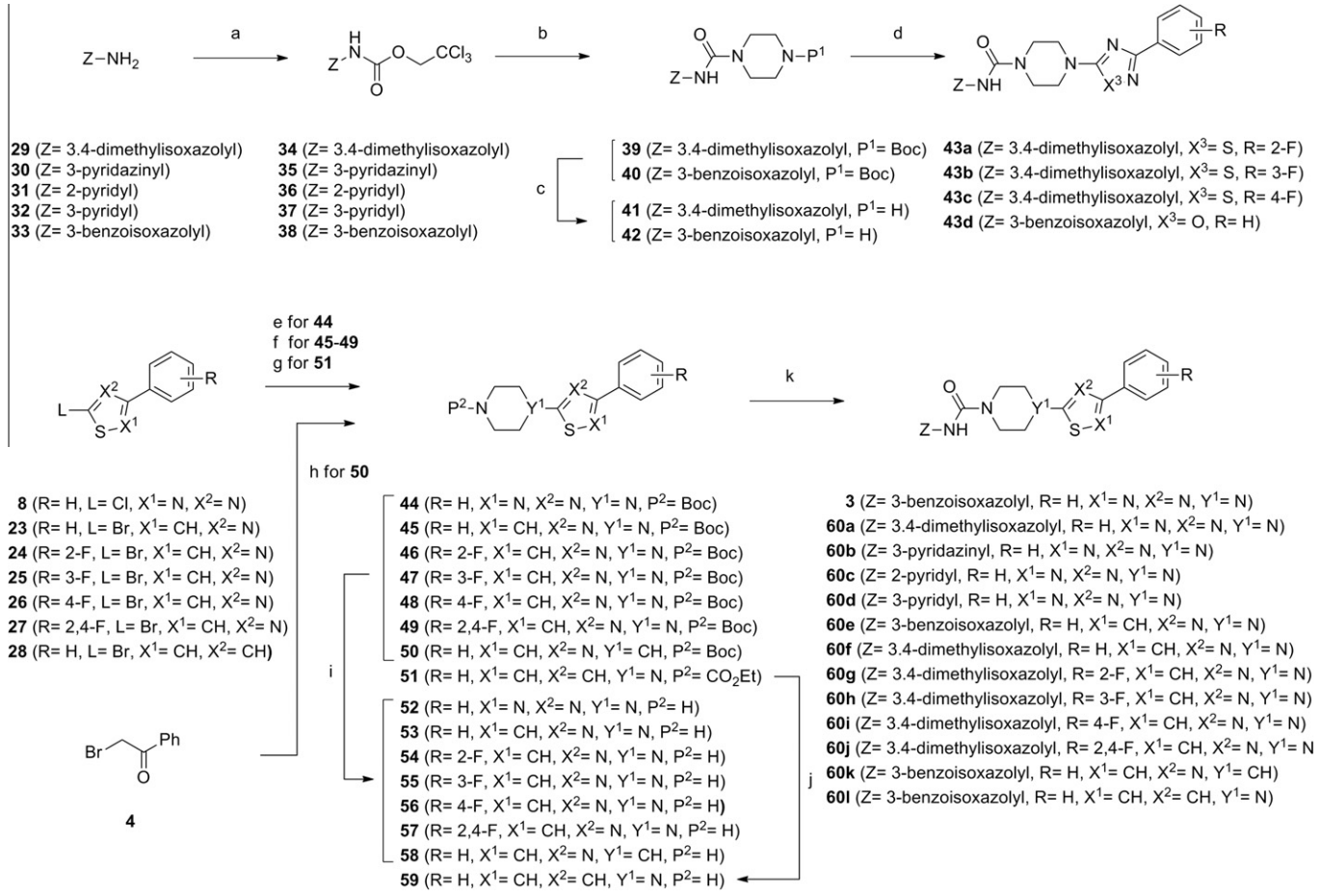
3. Results and discussion

The synthesized compounds were evaluated using in vitro and in vivo biological tests as follows. The FAAH inhibitory activity was measured by radioisotopic (RI) assay using human and rat FAAH enzyme fractions and the substrate anandamide [ethanolamine 1-^{3}H] (Tables 1–4). The pharmacokinetic (PK) profiles of the compounds were analyzed by rat cassette-dosing tests (Table 4). The brain concentration was measured from homogenized brain tissue 1 h after administration in rats (0.1 mg, i.v.) (Table 4). The in vivo therapeutic efficacy of the compounds was evaluated using the acetic acid-induced writhing test in mice, a representative pain model for the evaluation of compounds (Fig. 4).

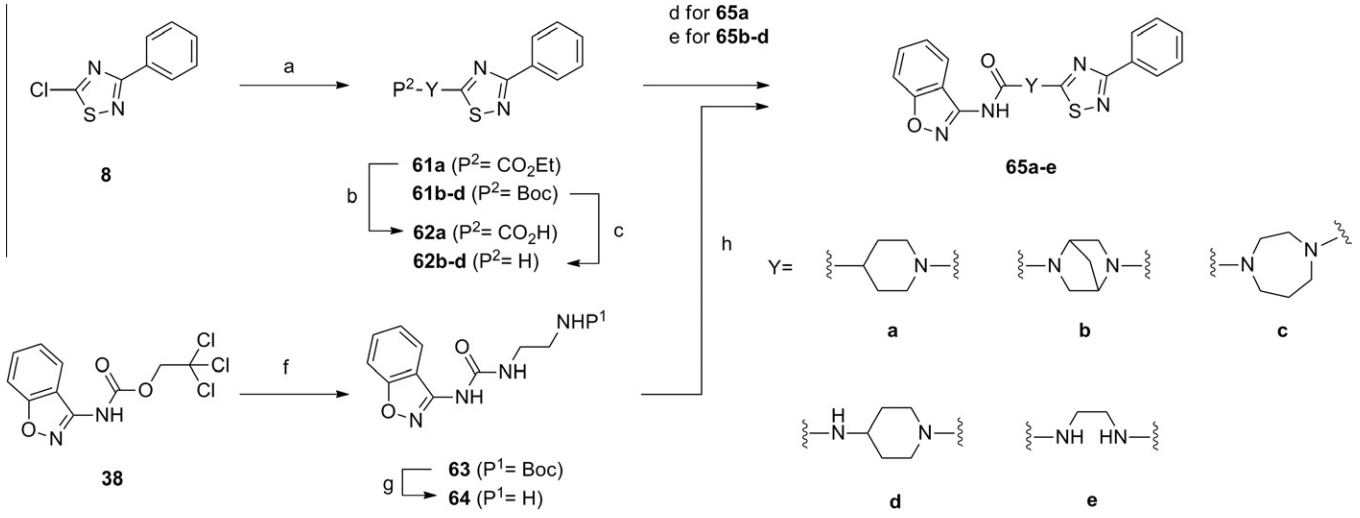
First, the replacement of the thiadiazole ring at **X** with other five-membered ring systems was examined, as shown in Table 1. The exchange of the sulfur atom for an oxygen atom in the thiadiazole ring diminished the inhibitory activity (**43d**). The thiazole derivative **60e**, in which the nitrogen atom at the 2-position of



Scheme 1. Synthesis of compounds **8**–**11** and **23**–**28**. Reagents and conditions: (a) KSCN, EtOH, 80 °C, 68–96%; (b) perchloromethyl mercaptan, NaOH, CH_2Cl_2, H_2O, 0 °C–room temp, 71–87%; (c) HBr, AcOH, 130 °C, 61–99%; (d) Br_2, AcOH.



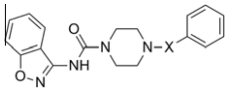
Scheme 2. Synthesis of compounds **43a**–**43d** and **60a**–**60l**. Reagents and conditions: (a) 2,2,2-trichloroethyl chloroformate, pyridine, THF, 0 °C, 9–64%; (b) *N*-Boc-piperazine, $^{i}Pr_2NEt$, DMSO, 70 °C, 33–41%; (c) TFA, room temp, 95–96%; (d) **9**, **10**, **11**, or 5-chloro-3-phenyl-1,2,4-oxadiazole, Et_3N, DMF, room temp, 16–72%; (e) *N*-Boc-piperazine, Et_3N, DMF, room temp, 57%; (f) *N*-Boc-piperazine, K_2CO_3, DMF, 120 °C, 52–86%; (g) *N*-ethoxycarbonylpiperazine, $Pd_2(dba)_3$, BINAP, NaO^{t}Bu, toluene, 100 °C, 5%; (h) *N*-Boc-4-aminothiocarbonylpiperidine, K_2CO_3, DMF, 110 °C, 55%; (i) 4 N HCl in EtOAc, room temp then 1 N NaOH, 67–97%; (j) 8 N NaOH, EtOH, 100 °C, 89%; (k) **34**, **35**, **36**, **37**, or **38**, $^{i}Pr_2NEt$, DMSO, 70 °C, 14–70%.



Scheme 3. Synthesis of compounds **65a**–**65e**. Reagents and conditions: (a) amines (R^1–Y–H), Et_3N, DMF, room temp, 70–99%; (b) 2 N NaOH, THF–EtOH, 80 °C, 89%; (c) 2 N HCl in MeOH, room temp then 1 N NaOH, 24–95%; (d) $(COCl)_2$, 3-amino-benzoisoxazole, pyridine, THF–DMF, room temp, 15%; (e) **38**, $^{i}Pr_2NEt$, DMSO, 70 °C, 31–54%; (f) $BocHN(CH_2)_2NH_2$, $^{i}Pr_2NEt$, DMSO, 70 °C, 87%; (g) 4 N HCl in EtOAc, room temp then 1 N NaOH, 84%; (h) **8**, Et_3N, NaI, MeCN, 90 °C, 23%.

the thiadiazole ring was replaced with CH, was equipotent to compound **3**. From our putative binding model, Thr488 is located near this area, and the 2-nitrogen atom of the thiadiazole or the 5-hydrogen of the thiazole ring are assumed to act as a hydrogen-bond acceptor/donor by interacting with the Thr488 residue. However, the removal of the nitrogen atom of the thiazole ring

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



Compds	X	Apparent FAAH IC_{50}[a] (nM) (Human/rat)
3		4.8/5.5
43d		87/130
60e		3.7/6.1
60l		110/290

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

decreased the activity (**60l**). These results indicated that the sulfur atom and the nitrogen atom at the 4-position of the thiadiazole ring were important for the FAAH inhibitory activity, and that the thiadiazole and thiazole ring were favorable for group **X**.

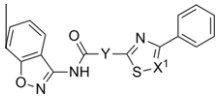
Table 2 shows the SAR for the central piperazine ring moiety **Y**. Replacement of the left-hand nitrogen atom with carbon resulted in a decrease in inhibitory activity (**65a**). Compound **65d**, with a secondary amine outside the piperidine ring, also showed a similar loss in activity. The thiazolylpiperidine compound **60k**, in which the right-hand nitrogen atom was replaced with carbon, resulted in a 10-fold loss in activity compared to compound **3**. Ring opening, ring expansion, or replacement with a bicyclic structure were not tolerated (**65e**, **65c**, **65b**). The results indicated that piperazine was favorable for the central ring **Y**.

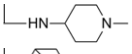
In the optimization of the terminal heterocycle **Z**, as shown in Table 3, the 3,4-dimethylisoxazolyl analog **60a** showed strong in vitro activity. In addition, notably improved solubility was observed for **60a** (1.6 μg/mL in pH 6.8 solution), presumably because of its increased hydrophilicity caused by the removal of the benzene ring part from the benzoisoxazole group. Introduction of a 3-pyridazinyl group instead of the 5-membered ring, produced compound **60b** which exhibited good potency and an additional increase in solubility (6.3 μg/mL in pH 6.8 solution). However, the replacement of the pyridazinyl group with a 2-pyridyl group diminished the activity (**60c**). On the other hand, the 3-pyridyl analog **60d** showed a potency comparable to that of compound **60b**. These results indicated that heteroaryl groups having a heteroatom at the meta position improved the inhibitory potency and solubility. A nitrogen atom at that position is assumed to act as a hydrogen-bond acceptor by interacting with Thr236. We selected the 3,4-dimethylisoxazolyl derivative **60a**, which showed the most promising activity, for further study, and investigated the substituent **R** on the phenyl ring.

In assessing the substituent **R**, we investigated whether the introduction of fluorine could result in increased potency as well as brain permeability by a strengthening of the interaction with the lipophilic site of FAAH. In addition to thiadiazole derivatives, thiazole analogs were evaluated, taking into account the comparable activities of **60e** and **3**. The results are shown in Table 4. The introduction of a 4-fluoro group resulted in a slight increase in activity in the series of thiadiazole derivatives (**43c** vs **60a**, **43a**, **43b**). Among the thiazole derivatives, an increase in brain concentration as well as drug exposure was achieved by fluorine introduction. In addition, the 2-fluoro and 4-fluoro groups (**60g** and **60i**) exhibited potent inhibitory activities. The thiazole derivatives showed more potent inhibitory activities along with higher exposures and bioavailabilities than the thiadiazole derivatives (**60a**, **43a**, **43b**, **43c** vs **60f**, **60g**, **60h**, **60i**). It is noteworthy that the replacement of thiadiazole into the thiazole ring led to great improvement of the solubility despite the increasing lipophilicity ($c\log P$ **60a**/**60f** = 2.4/3.1). This phenomenon was thought to be advantageous not only for the inhibitory activity, but also for brain penetration. Finally, we identified the 2,4-difluorophenyl derivative **60j** with extraordinarily potent activity for the FAAH enzyme (hFAAH/rFAAH IC_{50} = 0.43/0.46 nM) and sufficient drug exposure, brain concentration, and bioavailability. Compound **60j** was orally available, and exhibited a high brain concentration in mice (AUC = 16.7 μg h/mL, MRT = 3.7 h, brain concentration at 1 h = 5.86 μg/g at a dose of 10 mg/kg, po (n = 3)).

Compound **60j** was evaluated for its in vivo analgesic efficacy using the acetic acid-induced writhing test in mice. As shown in Figure 4, oral administration of compound **60j** exerted robust anti-nociceptive efficacy, which is defined as a decreased number of stretching movements in a dose-dependent manner. Further studies to evaluate the effects of compound **60j** in other various pain models are worth exploring the potential of compound **60j** as an analgesic agent.

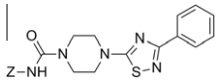
Table 2
Human and rat FAAH activities of derivatives modified at the central piperazine ring moiety



Compds	Y	X	Apparent FAAH IC_{50}[a] (nM) (Human/rat)	Compds	Y	X	Apparent FAAH IC_{50}[a] (nM) (Human/rat)
3		N	4.8/5.5	**65c**		N	>1000/>1000
65a		N	>1000/>1000	**65d**		N	>1000/>1000
60k		CH	43/55	**65b**		N	>1000/>1000
65e		N	>1000/>1000				

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

Table 3
Human and rat FAAH activities of derivatives modified at the terminal heterocycle



Compds	Z	Apparent FAAH IC_{50}[a] (nM) (Human/rat)	Solubility[b] (μg/mL)
3		4.8/5.5	0.09
60a		1.7/1.3	1.6
60b		2.9/3.3	6.3
60c		120/48	NT[c]
60d		2.4/3.8	4.9

[a] IC_{50} value were determined at a 30 min reaction.
[b] Solubility in pH 6.8 solution.
[c] Not tested.

These piperazine–urea-type derivatives are assumed to form a covalent bond with FAAH to inactivate the enzyme irreversibly. To confirm this with our compounds, we performed co-crystallization study of compound **60d** with rat FAAH. The co-crystal of compound **60d** with rat FAAH enzyme indicated the tight binding of **60d** with FAAH (Fig. 5). The bound complex is the carbamylated enzyme resulting from the attack of the catalytic Ser241 residue at the urea carbonyl carbon within the active site and the loss of the 3-aminopyridine as a leaving group. The piperazine ring forms a chair conformation, and the carbonyl oxygen interacts with the backbone nitrogen atoms in the 238–241 loop.

4. Conclusion

In the course of our exploration of novel FAAH inhibitors that are therapeutically effective against pain, we designed, synthesized, and evaluated a series of piperazine urea compounds. The removal of the benzene ring in the benzoisoxazole moiety improved the solubility. Replacement of the thiadiazole ring with thiazole led to increase of the FAAH inhibitory activity and a further improvement of solubility. The introduction of fluoro group on the phenyl ring enhanced activity along with increase of drug exposure and brain permeability to give compound **60j**. Oral administration of compound **60j** showed potent analgesic efficacy in the acetic acid-induced writhing test in mice. The results suggest that potent and selective FAAH inhibitors have potencial as beneficial analgesic agents.

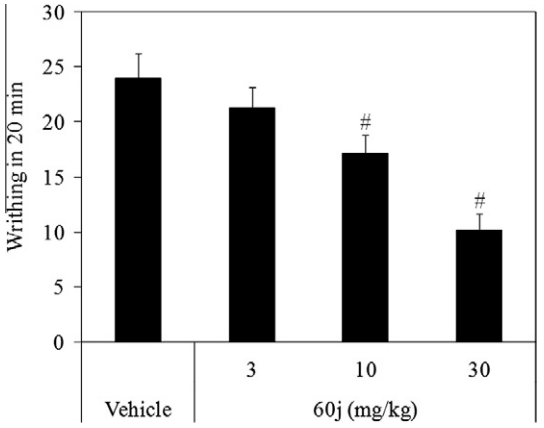


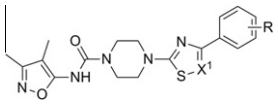
Figure 4. Analgesic effect of compound **60j** in acetic acid-induced writhing test. Compound **60j** (3, 10, 30 mg/kg) and vehicle were orally administered 60 min prior to acetic acid injection. Data are shown in mean SEM, $n = 10$, $^{\#}P < 0.025$ vs vehicle (Williams test).

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

Table 4
Profiles of the thiadiazole and thiazole derivatives



Compds	R	X^1	Apparent FAAH IC_{50}[a] (nM) (Human/rat)	Solubility[b] (μg/mL)	AUC[c] (ng·h/mL)	F[c] (%)	Brain conc.[d] (ng/g at 1 h)
60a	H	N	1.7/1.3	1.6	645	66	25
60f	H	CH	0.92/0.96	38	915	45	23
43a	2-F	N	2.1/1.2	1.9	415	31	4.6
60g	2-F	CH	0.67/0.75	3.0	398	98	103
43b	3-F	N	3.2/2.0	0.51	706	42	12
60h	3-F	CH	1.2/1.4	17	1329	91	101
43c	4-F	N	1.3/0.80	0.56	1040	70	17
60i	4-F	CH	0.86/0.71	2.7	2655	118	132
60j	2,4-F	CH	0.43/0.46	1.3	1309	98	82

[a] IC_{50} value were determined at a 30 min reaction.
[b] Solubility in pH 6.8 solution.
[c] Rat cassette dosing, 1 mg/kg, po.
[d] Rat 0.1 mg/kg, i.v. administration.

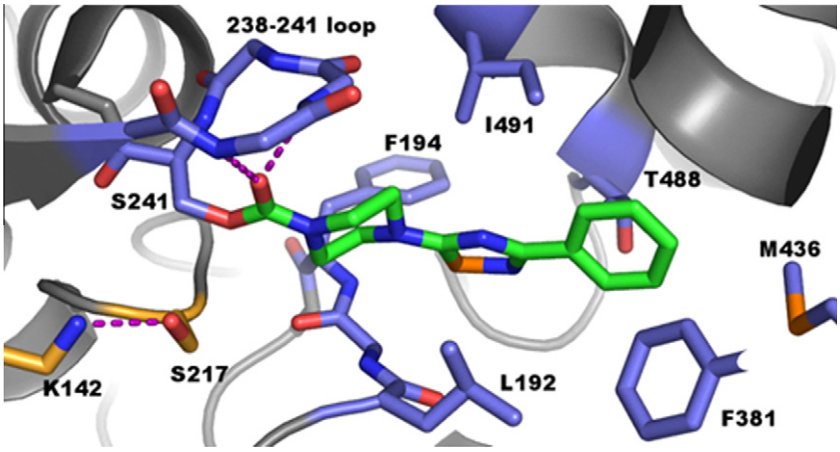


Figure 5. Co-crystallization study of compound **60d** with rat FAAH protein.

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. 5-Chloro-3-phenyl-1,2,4-thiadiazole (8)

To a stirred solution of benzamidine hydrochloride (25 g, 160 mmol) and perchloromethyl mercaptan (17.2 mL, 160 mmol) in CH_2Cl_2 (160 mL) was added a solution of NaOH (31.9 g, 798 mmol) in H_2O (65 mL) dropwise at 0 °C. The mixture was stirred at 0 °C for 1.0 h and at room temperature for 1.0 h. The organic layer was washed with water, dried over anhydrous $MgSO_4$, and concentrated in vacuo to give **8** (31.4 g) as a pale yellow oil. This product was used for next reaction without further purification.

5.1.2. 5-Chloro-3-(2-fluorophenyl)-1,2,4-thiadiazole (9)

To a stirred solution of 2-fluorobenzenecarboximidamide (1.38 g, 10.0 mmol) and perchloromethyl mercaptan (1.07 mL, 10.0 mmol) in CH_2Cl_2 (10 mL) was added a solution of NaOH (1.60 g, 40.0 mmol) in H_2O (4.0 mL) dropwise at 0 °C. The mixture was stirred at 0 °C for 1.0 h and at room temperature for 1.0 h. 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 **9** (1.88 g, 87%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.22–7.30 (2H, m), 7.44–7.52 (1H, m), 8.14–8.19 (1H, m).

5.1.3. 5-Chloro-3-(3-fluorophenyl)-1,2,4-thiadiazole (10)

Compound **10** was prepared in a manner similar to that described for **9** in 71% yield as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.15–7.22 (1H, m), 7.42–7.49 (1H, m), 7.93–7.97 (1H, m), 8.03–8.06 (1H, m).

5.1.4. 5-Chloro-3-(4-fluorophenyl)-1,2,4-thiadiazole (11)

Compound **11** was prepared in a manner similar to that described for **9** in 85% yield as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.12–7.19 (2H, m), 8.22–8.27 (2H, m).

5.1.5. 1-Phenyl-2-thiocyanatoethanone (17)

A mixture of 2-bromo-1-phenylethanone (10 g, 50.0 mmol), potassium thiocyanate (4.90 g, 50.0 mmol), and EtOH (80 mL) was stirred at 80 °C for 3.0 h. After cooling to room temperature, the mixture was diluted with water. The solid was collected by filtration and washed with 50% EtOH/water to give **17** (5.99 g, 68%) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.75 (2H, s), 7.51–7.56 (2H, m), 7.65–7.71 (1H, m), 7.93–7.97 (2H, m).

5.1.6. 1-(2-Fluorophenyl)-2-thiocyanatoethanone (18)

A mixture of 2-bromo-1-(2-fluorophenyl)ethanone (5.0 g, 23.0 mmol), potassium thiocyanate (2.24 g, 23.0 mmol), and EtOH (50 mL) was stirred at 80 °C for 2.0 h, diluted with water, and extracted with chloroform. The organic layer was washed with water, dried over anhydrous $MgSO_4$, and concentrated in vacuo to give **18** (4.29 g, 96%) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.66 (2H, d, J = 3.0 Hz), 7.19–7.25 (1H, m), 7.29–7.35 (1H, m), 7.62–7.69 (1H, m), 7.96–8.02 (1H, m).

5.1.7. 1-(3-Fluorophenyl)-2-thiocyanatoethanone (19)

Compound **19** was prepared in a manner similar to that described for **17** in 74% yield as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.70 (2H, s), 7.35–7.42 (1H, m), 7.50–7.57 (1H, m), 7.62–7.67 (1H, m), 7.71–7.74 (1H, m).

5.1.8. 1-(4-Fluorophenyl)-2-thiocyanatoethanone (20)

Compound **20** was prepared in a manner similar to that described for **17** in 83% yield as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.71 (2H, s), 7.18–7.22 (2H, m), 7.96–8.01 (2H, m).

5.1.9. 1-(2,4-Difluorophenyl)-2-thiocyanatoethanone (21)

Compound **21** was prepared in a manner similar to that described for **17** in 75% yield as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.61 (1H, s), 4.62 (1H, s), 6.92–7.09 (2H, m), 8.02–8.10 (1H, m).

5.1.10. 2-Bromo-4-phenylthiazole (23)

To a stirred solution of 1-(2-fluorophenyl)-2-thiocyanatoethanone (20.0 g, 113 mmol) in AcOH (200 mL) was added 25% HBr in

AcOH (200 mL) dropwise at room temperature. The mixture was stirred at 130 °C for 2.0 h and at room temperature for 1.0 h. The mixture was diluted with water, and extracted with chloroform. 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 **23** (15.2 g, 56%) as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.35–7.45 (4H, m), 7.83–7.87 (2H, m).

5.1.11. 2-Bromo-4-(2-fluorophenyl)thiazole (24)

Compound **24** was prepared in a manner similar to that described for **23** in 61% yield as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.10–7.35 (3H, m), 7.69 (1H, s), 8.14–8.19 (1H, m).

5.1.12. 2-Bromo-4-(3-fluorophenyl)thiazole (25)

Compound **25** was prepared in a manner similar to that described for **23** in 99% yield as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.04–7.05 (1H, m), 7.34–7.42 (1H, m), 7.43 (1H, s), 7.56–7.64 (2H, m).

5.1.13. 2-Bromo-4-(4-fluorophenyl)thiazole (26)

Compound **26** was prepared in a manner similar to that described for **23** in 92% yield as a pale yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.07–7.14 (2H, m), 7.34 (1H, s), 7.80–7.86 (2H, m).

5.1.14. 2-Bromo-4-(2,4-difluorophenyl)thiazole (27)

Compound **27** was prepared in a manner similar to that described for **23** in 74% yield as a pale yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.86–7.00 (2H, m), 7.62–7.63 (1H, m), 8.12–8.20 (1H, m).

5.1.15. 2,2,2-Trichloroethyl (3,4-dimethylisoxazol-5-yl)carbamate (34)

To a stirred solution of 5-amino-3,4-dimethylisoxazole (1.0 g, 8.92 mmol) and pyridine (0.873 mL, 10.7 mmol) in THF (30 mL) was added 2,2,2-trichloroethyl chloroformate (1.48 mL, 10.7 mmol) dropwise at 0 °C. 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 purified by silica gel column chromatography (hexane–EtOAc) to give **34** (1.61 g, 63%) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.92 (3H, s), 2.22 (3H, s), 4.82 (2H, s), 7.40 (1H, br s).

5.1.16. 2,2,2-Trichloroethyl pyridazin-3-ylcarbamate (35)

Compound **35** was prepared in a manner similar to that described for **34** in 9% yield as a yellow powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.87 (2H, s), 7.52 (1H, dd, J = 8.7, 5.1 Hz), 8.25 (1H, dd, J = 1.5, 8.7 Hz), 8.31 (1H, br s), 8.95 (1H, dd, J = 1.5, 5.1 Hz).

5.1.17. 2,2,2-Trichloroethyl pyridazin-2-ylcarbamate (36)

To a stirred solution of pyridin-2-amine (1.0 g, 10.6 mmol) and pyridine (1.01 mL, 12.7 mmol) in THF (35 mL) was added 2,2,2-trichloroethyl chloroformate (1.76 mL, 12.7 mmol) dropwise at 0 °C. The mixture was stirred at 0 °C for 1.5 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 triturated with Et_2O–hexane to give **36** (1.76 g, 62%) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.88 (2H, s), 7.01–7.05 (1H, m), 7.71–7.76 (1H, m), 8.03 (1H, d, J = 8.4 Hz), 8.48–8.51 (1H, m).

5.1.18. 2,2,2-Trichloroethyl pyridin-3-ylcarbamate (37)

Compound **37** was prepared in a manner similar to that described for **36** in 64% yield as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.85 (2H, s), 7.31 (1H, dd, J = 4.8, 8.4 Hz), 8.01 (1H, br s), 8.07–8.10 (1H, m), 8.40 (1H, d, J = 4.8 Hz), 8.58–8.59 (1H, m).

5.1.19. 2,2,2-Trichloroethyl benzo[*d*]isoxazol-3-ylcarbamate (38)

Compound **38** was prepared in a manner similar to that described for **36** in 58% yield as an off-white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 4.91 (2H, s), 7.29–7.39 (1H, m), 7.47–7.64 (2H, m), 7.88 (1H, br s), 8.16 (1H, d, J = 7.9 Hz).

5.1.20. *tert*-Butyl 4-[(3,4-dimethylisoxazol-5-yl)carbamoyl]piperazine-1-carboxylate (39)

A mixture of *N*-Boc-piperazine (5.0 g, 26.8 mmol), 2,2,2-trichloroethyl (3,4-dimethylisoxazol-5-yl)carbamate (3.85 g, 13.4 mmol), *N*-ethyldiisopropylamine (2.33 mL, 13.4 mmol), and DMSO (50 mL) was stirred at 70 °C for 2.0 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 **39** (1.80 g, 41%) as colorless crystals. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.48 (9H, s), 1.87 (3H, s), 2.19 (3H, s), 3.49 (8H, s), 6.81 (1H, br s).

5.1.21. *tert*-Butyl 4-(benzo[*d*]isoxazol-3-ylcarbamoyl)piperazine-1-carboxylate (40)

Compound **40** was prepared in a manner similar to that described for **39** in 33% yield as colorless crystals. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.50 (9H, s), 3.56–3.66 (8H, m), 7.27–7.32 (1H, m), 7.46–7.58 (2H, m), 8.05 (1H, d, J = 8.4 Hz), 8.37 (1H, s).

5.1.22. *N*-(3,4-Dimethylisoxazol-5-yl)piperazine-1-carboxamide 2,2,2-trifluoroacetate (41)

A mixture of *tert*-butyl 4-[(3,4-dimethylisoxazol-5-yl)carbamoyl]piperazine-1-carboxylate (1.00 g, 3.08 mmol) and trifluoroacetic acid (20 mL) was stirred at room temperature for 2.0 h, and then the solvent was concentrated in vacuo to give **41** (1.00 g, 96%) as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.76 (3H, s), 2.13 (3H, s), 3.17 (4H, br s), 3.63 (4H, br s), 8.89 (2H, br s), 9.38 (1H, br s).

5.1.23. *N*-(Benzo[*d*]isoxazol-3-yl)piperazine-1-carboxamide 2,2,2-trifluoroacetate (42)

A mixture of *tert*-butyl 4-(benzo[*d*]isoxazol-3-ylcarbamoyl)piperazine-1-carboxylate (16.5 g, 47.6 mmol) and trifluoroacetic acid (200 mL) was stirred at room temperature for 2.0 h, and then the solvent was concentrated in vacuo. The residue was recrystallized from MeOH–Et_2O to give **42** (16.3 g, 95%) as a solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.18–3.22 (4H, m), 3.72–3.75 (4H, m), 7.30–7.35 (1H, m), 7.59–7.67 (2H, m), 7.86 (1H, d, J = 8.4 Hz), 8.99 (2H, br s), 10.11 (1H, s).

5.1.24. *N*-(3,4-Dimethylisoxazol-5-yl)-4-[3-(2-fluorophenyl)-1,2,4-thiadiazol-5-yl]piperazine-1-carboxamide (43a)

A mixture of *N*-(3,4-dimethylisoxazol-5-yl)piperazine-1-carboxamide trifluoroacetate (297 mg, 0.707 mmol), 5-chloro-3-(2-fluorophenyl)-1,2,4-thiadiazole (152 mg, 0.707 mmol), triethylamine (0.491 ml, 3.53 mmol), and DMF (3.0 mL) was stirred at room temperature for 3.0 h, poured into water, and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was recrystallized from EtOAc–hexane to give **43a** (44.0 mg, 16%) as colorless crystals, mp 239–240 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.76 (3H, s), 2.13 (3H, s), 3.63 (8H, br s), 7.28–7.35 (2H, m),

7.49–7.56 (1H, m), 8.00–8.06 (1H, m), 9.34 (1H, s). Analytical HPLC showed 100% purity.

5.1.25. *N*-(3,4-Dimethylisoxazol-5-yl)-4-[3-(3-fluorophenyl)-1,2,4-thiadiazol-5-yl]piperazine-1-carboxamide (43b)

Compound **43b** was prepared in a manner similar to that described for **43a** in 45% yield as colorless crystals, mp 219–220 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.88 (3H, s), 2.20 (3H, s), 3.66–3.75 (8H, m), 7.08–7.15 (1H, m), 7.36–7.44 (1H, m), 7.86–7.91 (1H, m), 7.96–8.00 (1H, m), 9.05 (1H, s). Analytical HPLC showed 100% purity.

5.1.26. *N*-(3,4-Dimethylisoxazol-5-yl)-4-[3-(4-fluorophenyl)-1,2,4-thiadiazol-5-yl]piperazine-1-carboxamide (43c)

Compound **43c** was prepared in a manner similar to that described for **43a** in 20% yield as colorless crystals, mp 220–221 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.77 (3H, s), 2.13 (3H, s), 3.64 (8H, s), 7.28–7.34 (2H, m), 8.13–8.18 (2H, m), 9.34 (1H, s). Analytical HPLC showed 98.2% purity.

5.1.27. *N*-(Benzo[*d*]isoxazol-3-yl)-4-(3-phenyl-1,2,4-oxadiazol-5-yl)piperazine-1-carboxamide (43d)

Compound **43d** was prepared in a manner similar to that described for **43a** in 72% yield as colorless crystals, mp 215–216 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.72 (8H, s), 7.30–7.35 (1H, m), 7.49–7.67 (5H, m), 7.85–7.94 (3H, m), 10.09 (1H, s). Anal. Calcd for $C_{20}H_{18}N_6O_3$: C, 61.53; H, 4.65; N, 21.53. Found: C, 61.68; H, 4.70; N, 21.42.

5.1.28. *tert*-Butyl 4-(3-phenyl-1,2,4-thiadiazol-5-yl)piperazine-1-carboxylate (44)

A mixture of *N*-Boc-piperazine (29.7 g, 160 mmol), 5-chloro-3-phenyl-1,2,4-thiadiazole (31.4 g, 160 mmol), triethylamine (89.0 ml, 638 mmol), and DMF (320 mL) was stirred at room temperature for 2.0 h, poured into water, and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane–EtOAc) to give **44** (31.6 g, 57% from **8**) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.50 (9H, s), 3.60 (8H, s), 7.38–7.49 (3H, m), 8.14–8.24 (2H, m).

5.1.29. *tert*-Butyl 4-(4-phenylthiazol-2-yl)piperazine-1-carboxylate (45)

A mixture of *N*-Boc-piperazine (25.4 g, 136 mmol), 2-bromo-4-phenylthiazole (16.4 g, 68.2 mmol), potassium carbonate (9.43 g, 68.2 mmol), and DMF (230 mL) was stirred at 120 °C for 8.0 h, poured into water, and extracted with EtOAc. The organic layer was washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane–EtOAc) to give **45** (14.6 g, 62%) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.49 (9H, s), 3.51–3.59 (8H, m), 6.79 (1H, s), 7.27–7.39 (3H, m), 7.80– 7.83 (2H, m).

5.1.30. *tert*-Butyl 4-[4-(2-fluorophenyl)thiazol-2-yl]piperazine-1-carboxylate (46)

Compound **46** was prepared in a manner similar to that described for **45** in 80% yield as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.49 (9H, s), 3.48–3.61 (8H, m), 7.05–7.27 (4H, m), 8.10–8.16 (1H, m).

5.1.31. *tert*-Butyl 4-[4-(3-fluorophenyl)thiazol-2-yl]piperazine-1-carboxylate (47)

Compound **47** was prepared in a manner similar to that described for **45** in 69% yield as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.49 (9H, s), 3.51–3.61 (8H, m), 6.82 (1H, s), 6.94–7.00 (1H, m), 7.29–7.36 (1H,m), 7.53–7.60 (2H,m).

5.1.32. *tert*-Butyl 4-[4-(4-fluorophenyl)thiazol-2-yl]piperazine-1-carboxylate (48)

Compound **48** was prepared in a manner similar to that described for **45** in 52% yield as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.49 (9H, s), 3.50–3.60 (8H, m), 6.71 (1H, s), 7.02–7.08 (2H, m), 7.77–7.82 (2H, m).

5.1.33. *tert*-Butyl 4-[4-(2,4-difluorophenyl)thiazol-2-yl]piperazine-1-carboxylate (49)

Compound **49** was prepared in a manner similar to that described for **45** in 86% yield as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.49 (9H, s), 3.48–3.60 (8H, m), 6.81–6.95 (2H, m), 7.02–7.03 (1H, m), 8.08–8.16 (1H, m).

5.1.34. *tert*-Butyl 4-(4-phenylthiazol-2-yl)piperidine-1-carboxylate (50)

A mixture of *tert*-butyl 4-carbamothioylpiperidine-1-carboxylate (1.0 g, 7.24 mmol), 2-bromo-1-phenylethanone (1.58 g, 7.96 mmol), potassium carbonate (1.0 g, 7.24 mmol), and DMF (30 mL) was stirred at 110 °C for 1.5 h, poured into water, and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue purified by silica gel column chromatography (hexane–EtOAc) to give **50** (1.38 g, 55%) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.46 (9H, s), 1.69–1.86 (2H, m), 2.13–2.18 (2H, m), 2.88–2.96 (2H, m), 3.17–3.27 (1H, m), 4.19 (2H, br s), 7.29–7.51 (4H, m), 7.86–7.89 (2H, m).

5.1.35. Ethyl 4-(4-phenylthiophen-2-yl)piperazine-1-carboxylate (51)

To a stirred solution of 3-phenylthiophene (5.00 g, 32.0 mmol) in AcOH (65 mL) was added a solution of bromine (5.00 g, 32.0 mmol) in AcOH (50 mL) dropwise at room temperature, and the mixture was refluxed for 5.0 h. After cooling to room temperature, the mixture was poured into water, and extracted with Et_2O. The organic layer was washed with sat. aq $NaHCO_3$, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane–EtOAc) to give the mixture of 2-bromo-4-phenylthiophene and 2-bromo-3-phenylthiophene (6.00 g) as an oil. The obtained compound was dissolved in toluene (50 mL), and ethyl piperazine-1-carboxylate (3.96 g, 25.0 mmol), sodium *tert*-butoxide (3.48 g, 36.2 mmol), 2,2′-bis(diphenylphosphino)-1,1′-binaphthyl (468 mg, 0.750 mmol), tris(dibenzylideneacetone)dipalladium (0) (544 mg, 0.594 mmol) were added to the mixture. The mixture was stirred at 100 °C for 48 h, and solid was removed by filtration, and the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane–EtOAc) to give **51** (365 mg, 5% from **22**) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.29 (3H, t, J = 7.2 Hz), 3.14–3.17 (4H, m), 3.63–3.67 (4H, m), 4.17 (2H, q, J = 7.2 Hz), 6.47 (1H, d, J = 1.8 Hz), 6.78 (1H, d, J = 1.8 Hz), 7.26–7.30 (1H, m), 7.34–7.40 (2H, m), 7.52–7.56 (2H, m).

5.1.36. 3-Phenyl-5-(piperazin-1-yl)-1,2,4-thiadiazole (52)

To a stirred solution of *tert*-butyl 4-(4-phenylthiazol-2-yl)piperazine-1-carboxylate (31.6 g, 91.3 mmol) in EtOAc (300 mL) was added 4 N HCl in EtOAc (300 mL) dropwise at room temperature. After stirring at room temperature overnight, the mixture was diluted with hexane (600 mL). The solid was collected by filtration, and suspended in water, neutralized with sat. aq $NaHCO_3$. The resulting precipitate was collected by filtration and washed with water to give **52** (21.7 g, 97%) as a colorless powder. ^{1}H NMR

(300 MHz, DMSO-d_6) δ: 2.77–2.91 (4H, m), 3.41–3.57 (4H, m), 7.42–7.51 (3H, m), 8.06–8.16 (2H, m).

5.1.37. 4-Phenyl-2-(piperazin-1-yl)thiazole (53)

To a stirred solution of *tert*-butyl 4-(4-phenylthiazol-2-yl)piperazine-1-carboxylate (14.1 g, 40.7 mmol) in EtOAc (220 mL) was added 4 N HCl in EtOAc (100 mL) dropwise at room temperature. After stirring at room temperature for 4.0 h, the mixture was concentrated in vacuo. The residue was dissolved in water, neutralized with 1 N NaOH, and extracted with chloroform. The organic layer was dried over anhydrous $MgSO_4$, and concentrated in vacuo to give **53** (10.1 g, quant.) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.99–3.03 (4H, m), 3.51–3.54 (4H, m), 6.77 (1H, s), 7.24–7.40 (3H, m), 7.82–7.85 (2H, m).

5.1.38. 4-(2-Fluorophenyl)-2-(piperazin-1-yl)thiazole (54)

Compound **54** was prepared in a manner similar to that described for **53** in 67% yield as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.99–3.02 (4H, m), 3.50–3.54 (4H, m), 7.05–7.25 (4H, m), 8.12–8.18 (1H, m).

5.1.39. 4-(3-Fluorophenyl)-2-(piperazin-1-yl)thiazole (55)

Compound **55** was prepared in a manner similar to that described for **53** in 86% yield as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.99–3.03 (4H, m), 3.50–3.54 (4H, m), 6.79 (1H, s), 6.90–6.99 (1H, m), 7.28–7.35 (1H, m), 7.53–7.61 (2H, m).

5.1.40. 4-(4-Fluorophenyl)-2-(piperazin-1-yl)thiazole (56)

Compound **56** was prepared in a manner similar to that described for **53** in 67% yield as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.94–2.99 (4H, m), 3.45–3.50 (4H, m), 6.64 (1H, s), 6.96–7.05 (2H, m), 7.24–7.80 (2H, m).

5.1.41. 4-(2,4-Difluorophenyl)-2-(piperazin-1-yl)thiazole (57)

Compound **57** was prepared in a manner similar to that described for **53** in 74% yield as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.99–3.02 (4H, m), 3.49–3.53 (4H, m), 6.81–6.94 (2H, m), 6.99–7.00 (1H, m), 8.09–8.17 (1H, m).

5.1.42. 4-Phenyl-2-(piperidin-4-yl)thiazole (58)

Compound **58** was prepared in a manner similar to that described for **53** in 98% yield as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.70–1.83 (2H, m), 2.14–2.19 (2H, m), 2.74–2.83 (2H, m), 3.15–3.24 (3H, m), 7.28–7.44 (4H, m), 7.86–7.90 (2H, m).

5.1.43. 1-(4-Phenylthiophen-2-yl)piperazine (59)

To a stirred solution of ethyl 4-(4-phenylthiophen-2-yl)piperazine-1-carboxylate (350 mg, 1.11 mmol) in EtOH (10 mL) was added 8 N NaOH (5.0 mL, 40.0 mmol) dropwise at room temperature. After stirring at 100 °C for 3.0 h, the mixture was concentrated in vacuo. The residue was diluted with water, and extracted with chloroform. The organic layer was dried over anhydrous $MgSO_4$, and concentrated in vacuo to give **59** (240 mg, 89%) as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.03–3.06 (4H, m), 3.15–3.18 (4H, m), 6.42 (1H, d, J = 1.8 Hz), 6.74 (1H, d, J = 1.8 Hz), 7.26–7.29 (1H, m), 7.34–7.39 (2H, m), 7.54–7.56 (2H, m).

5.1.44. *N*-(3,4-Dimethylisoxazol-5-yl)-4-(3-phenyl-1,2,4-thiadiazol-5-yl)piperazine-1-carboxamide (60a)

A mixture of 3-phenyl-5-(piperazin-1-yl)-1,2,4-thiadiazole (212 mg, 0.738 mmol), 2,2,2-trichloroethyl (3,4-dimethylisoxazol-5-yl)carbamate (200 mg, 0.812 mmol), *N*-ethyldiisopropylamine (0.141 mL, 0.812 mmol), and DMSO (2.5 mL) was stirred at 70 °C for 3.0 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 THF–hexane to give **60a** (125 mg, 44%) as colorless crystals, mp 233–234 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.86 (3H, s), 2.19 (3H, s), 3.65–3.68 (4H, m), 3.73–3.76 (4H, m), 7.40–7.44 (3H, m), 8.15–8.18 (2H, m), 9.24 (1H, s). Anal. Calcd for $C_{18}H_{20}N_6O_2S$: C, 56.23; H, 5.24; N, 21.86. Found: C, 56.02; H, 5.21; N, 21.53.

5.1.45. 4-(3-Phenyl-1,2,4-thiadiazol-5-yl)-*N*-(pyridazin-3-yl)piperazine-1-carboxamide (60b)

A mixture of 2,2,2-trichloroethyl pyridazin-3-ylcarbamate (200 mg, 0.793 mmol), 1-(3-phenyl-1,2,4-thiadiazol-5-yl)piperazine (182 mg, 0.739 mmol), *N*-ethyldiisopropylamine (0.129 ml, 0.739 mmol), and DMSO (3.0 mL) was stirred at 70 °C overnight. The reaction mixture was poured into water and a solid was collected by filtration. This was recrystallized from EtOAc to give **60b** (117 mg, 43%) as off-white crystals, mp 185–186 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.65–3.73 (8H, m), 7.46–7.50 (3H, m), 7.59 (1H, dd, J = 9.0, 5.4 Hz), 8.01 (1H, dd, J = 1.5, 9.0 Hz), 8.11–8.14 (2H, m), 8.86 (1H, dd, J = 5.4, 1.5 Hz), 10.11 (1H, br s). Anal. Calcd for $C_{17}H_{17}N_7OS{\cdot}0.2H_2O$: C, 55.03; H, 4.73; N, 26.43. Found: C, 55.21; H, 4.67; N, 26.08.

5.1.46. 4-(3-Phenyl-1,2,4-thiadiazol-5-yl)-*N*-(pyridazin-2-yl)piperazine-1-carboxamide (60c)

A mixture of 3-phenyl-5-(piperazin-1-yl)-1,2,4-thiadiazole (200 mg, 0.738 mmol), 2,2,2-trichloroethyl pyridazin-2-ylcarbamate (271 mg, 0.610 mmol), *N*-ethyldiisopropylamine (0.129 mL, 0.738 mmol), and DMSO (2.5 mL) was stirred at 70 °C for 3.0 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 purified by silica gel column chromatography (hexane–EtOAc), and recrystallized from EtOAc–hexane to give **60c** (124 mg, 55%) as colorless crystals, mp 162–163 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.69–3.72 (8H, m), 6.96–7.02 (1H, m), 7.39–7.44 (4H, m), 7.64–7.70 (1H, m), 7.99 (1H, d, J = 8.1 Hz), 8.15–8.22 (3H, m). Anal. Calcd for $C_{18}H_{18}N_6OS$: C, 59.00; H, 4.95; N, 22.93. Found: C, 58.96; H, 4.95; N, 22.92.

5.1.47. 4-(3-Phenyl-1,2,4-thiadiazol-5-yl)-*N*-(pyridin-3-yl)piperazine-1-carboxamide hydrochloride (60d)

A mixture of 3-phenyl-5-(piperazin-1-yl)-1,2,4-thiadiazole (8.0 g, 32.5 mmol), 2,2,2-trichloroethyl pyridin-3-ylcarbamate (9.63 g, 35.7 mmol), *N*-ethyldiisopropylamine (6.20 mL, 35.7 mmol), and DMSO (108 mL) was stirred at 70 °C overnight, 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 (EtOAc), and recrystallized from EtOAc–hexane to give 4-(3-phenyl-1,2,4-thiadiazol-5-yl)-*N*-(pyridin-3-yl)piperazine-1-carboxamide. This product (5.10 g) was dissolved in THF (600 mL), and 4 N HCl in EtOAc (120 mL) was added. The mixture was stirred at room temperature for 1.0 h and concentrated in vacuo. The residue was recrystallized from MeOH–Et_2O to give **60d** (5.46 g, 42%) as colorless crystals, mp 198–199 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.68–3.71 (4H, m), 3.82–3.86 (4H, m), 7.40–7.44 (3H, m), 7.85–7.90 (1H, m), 8.11–8.16 (2H, m), 8.42 (1H, d, J = 5.4 Hz), 8.69–8.73 (1H, m), 9.29 (1H, m), 10.06 (1H, s). Anal. Calcd for $C_{18}H_{19}ClN_6OS$: C, 53.66; H, 4.75; N, 20.86; Cl, 8.80. Found: C, 53.54; H, 4.76; N, 20.77; Cl, 8.71.

5.1.48. *N*-(Benzo[*d*]isoxazol-3-yl)-4-(4-phenylthiazol-2-yl)piperazine-1-carboxamide (60e)

Compound **60e** was prepared in a manner similar to that described for **60a** in 70% yield as off-white crystals, mp 213–214 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.58–3.60 (4H, m), 3.62–3.72

(4H, m), 7.27–7.43 (5H, m), 7.58–7.66 (2H, m), 7.85–7.89 (3H, m), 10.07 (1H, s). Anal. Calcd for $C_{21}H_{19}N_5O_2S$: C, 62.21; H, 4.72; N, 17.27. Found: C, 62.18; H, 4.85; N, 17.02.

5.1.49. *N*-(3,4-Dimethylisoxazol-5-yl)-4-(4-phenylthiazol-2-yl)piperazine-1-carboxamide (60f)

Compound **60f** was prepared in a manner similar to that described for **60c** in 14% yield as colorless crystals, mp 185–186 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.89 (3H, s), 2.21 (3H, s), 3.66 (8H, s), 6.63 (1H, br s), 6.83 (1H, s), 7.29–7.31 (1H, m), 7.36–7.41 (2H, m), 7.82–7.84 (2H, m). Anal. Calcd for $C_{19}H_{21}N_5O_2S$: C, 59.51; H, 5.52; N, 18.26. Found: C, 59.50; H, 5.52; N, 18.30.

5.1.50. *N*-(3,4-Dimethylisoxazol-5-yl)-4-[4-(2-fluorophenyl)thiazol-2-yl]piperazine-1-carboxamide (60g)

Compound **60g** was prepared in a manner similar to that described for **60c** in 38% yield as colorless crystals, mp 179–180 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.89 (3H, s), 2.20 (3H, s), 3.63–3.69 (8H, m), 6.71 (1H, s), 7.07–7.26 (4H, m), 8.10–8.16 (1H, m). Anal. Calcd for $C_{19}H_{20}FN_5O_2S$: C, 56.84; H, 5.02; N, 17.44. Found: C, 56.53; H, 5.07; N, 17.04.

5.1.51. *N*-(3,4-Dimethylisoxazol-5-yl)-4-[4-(3-fluorophenyl)thiazol-2-yl]piperazine-1-carboxamide (60h)

Compound **60h** was prepared in a manner similar to that described for **60c** in 43% yield as colorless crystals, mp 144–145 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.87 (3H, s), 2.19 (3H, s), 3.59–3.69 (8H, m), 6.84 (1H, s), 6.94–7.01 (1H, m), 7.07 (1H, br s), 7.29–7.37 (1H, m), 7.52–7.60 (2H, m). Anal. Calcd for $C_{19}H_{20}FN_5O_2S$: C, 56.84; H, 5.02; N, 17.44. Found: C, 56.85; H, 5.04; N, 17.32.

5.1.52. *N*-(3,4-Dimethylisoxazol-5-yl)-4-[4-(4-fluorophenyl)thiazol-2-yl]piperazine-1-carboxamide (60i)

Compound **60i** was prepared in a manner similar to that described for **60c** in 50% yield as colorless crystals, mp 183–184 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.89 (3H, s), 2.20 (3H, s), 3.62–3.69 (8H, m), 6.72 (1H, s), 6.75 (1H, s), 7.03–7.09 (2H, m), 7.77–7.82 (2H, m). Anal. Calcd for $C_{19}H_{20}FN_5O_2S{\cdot}0.1H_2O$: C, 56.33; H, 5.00; N, 17.29. Found: C, 56.24; H, 5.03; N, 17.01.

5.1.53. 4-[4-(2,4-Difluorophenyl)thiazol-2-yl]-*N*-(3,4-dimethylisoxazol-5-yl)piperazine-1-carboxamide (60j)

Compound **60j** was prepared in a manner similar to that described for **60c** in 34% yield as colorless crystals, mp 166–167 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.89 (3H, s), 2.20 (3H, s), 3.61–3.70 (8H, m), 6.72 (1H, br s), 6.83–6.96 (2H, m), 7.05–7.06 (1H, m), 8.08–8.16 (1H, m). Anal. Calcd for $C_{19}H_{19}F_2N_5O_2S$: C, 54.41; H, 4.57; N, 16.70. Found: C, 54.40; H, 4.51; N, 16.96.

5.1.54. *N*-(Benzo[*d*]isoxazol-3-yl)-4-(4-phenylthiazol-2-yl)piperidine-1-carboxamide (60k)

Compound **60k** was prepared in a manner similar to that described for **60c** in 59% yield as off-white crystals, mp 129–130 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.71–1.82 (2H, m), 2.14–2.18 (2H, m), 3.09–3.16 (2H, m), 3.34–3.38 (1H, m), 4.24–4.29 (2H, m), 7.29–7.36 (2H, m), 7.42–7.47 (2H, m), 7.58–7.65 (2H, m), 7.84 (1H, d, J = 7.8 Hz), 7.95–7.98 (2H, m), 8.02 (1H, s), 7.94 (1H, s). Anal. Calcd for $C_{22}H_{20}N_4O_2S$: C, 65.33; H, 4.98; N, 13.85. Found: C, 65.04; H, 4.93; N, 13.68.

5.1.55. *N*-(Benzo[*d*]isoxazol-3-yl)-4-(4-phenylthiophen-2-yl)piperazine-1-carboxamide (60l)

Compound **60l** was prepared in a manner similar to that described for **60c** in 21% yield as colorless crystals, mp 179–180 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.23 (4H, br s), 3.71 (4H, br s), 6.72 (1H, s), 7.11 (1H, s), 7.24–7.41 (4H, m), 7.58–7.67 (4H, m), 7.84 (1H, d, J = 8.1 Hz), 10.04 (1H, s). Anal. Calcd for $C_{22}H_{20}N_4SO_2{\cdot}0.1H_2O$: C, 65.04; H, 5.01; N, 13.79. Found: C, 65.12; H, 4.80; N, 13.48.

5.1.56. Ethyl 1-(3-phenyl-1,2,4-thiadiazol-5-yl)piperidine-4-carboxylate (61a)

A mixture of ethyl piperidine-4-carboxylate (0.782 mL, 5.08 mmol), 5-chloro-3-phenyl-1,2,4-thiadiazole (1.00 g, 5.08 mmol), triethylamine (0.708 mL, 5.08 mmol), and DMF (10 mL) was stirred at room temperature for 30 min, poured into water, and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was recrystallized from EtOAc–hexane to give **61a** (1.22 g, 76%) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.28 (3H, t, J = 7.2 Hz), 1.82–1.95 (2H, m), 2.04–2.11 (2H, m), 2.55–2.63 (1H, m), 3.26–3.35 (2H, m), 3.95–4.00 (2H, m), 4.18 (2H, q, J = 7.2 Hz), 7.39–7.44 (3H, m), 8.17–8.20 (2H, m).

5.1.57. *tert*-Butyl 4-(4-phenylthiazol-2-yl)-1,4-diazepane-1-carboxylate (61b)

Compound **61b** was prepared in a manner similar to that described for **61a** in 89% yield as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.44 (9H, s), 2.01–2.09 (2H, m), 3.37–3.49 (2H, m), 3.66–3.73 (5H, m), 3.90 (1H, br s), 7.39–7.44 (3H, m), 8.16–8.21 (2H, m).

5.1.58. *tert*-Butyl 5-(3-phenyl-1,2,4-thiadiazol-5-yl)-2,5-diazabicyclo[2.2.1]heptane-2-carboxylate (61c)

Compound **61c** was prepared in a manner similar to that described for **61a** in 70% yield as a colorless powder, ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.44–1.48 (9H, m), 2.05 (2H, br s), 3.44–3.63 (4H, m), 4.06–4.74 (2H, m), 7.40–7.45 (3H, m), 8.18–8.20 (2H, m).

5.1.59. *tert*-Butyl [1-(3-phenyl-1,2,4-thiadiazol-5-yl)piperidin-4-yl]carbamate (61d)

Compound **61d** was prepared in a manner similar to that described for **61a** in 99% yield as a colorless powder, ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.46 (9H, s), 1.51–1.61 (2H, m), 2.07–2.12 (2H, m), 3.26–3.35 (2H, m), 3.74 (1H, br s), 3.96–4.00 (2H, m), 4.50 (1H, br s), 7.40–7.45 (3H, m), 8.15–8.19 (2H, m).

5.1.60. 1-(3-Phenyl-1,2,4-thiadiazol-5-yl)piperidine-4-carboxylic acid (62a)

To a stirred solution of ethyl 1-(3-phenyl-1,2,4-thiadiazol-5-yl)piperidine-4-carboxylate (1.12 g, 3.53 mmol) in THF (20 mL) and EtOH (12 mL) was added 2 N NaOH (4.0 mL, 8.00 mmol) dropwise at room temperature. After stirring at 80 °C for 1 h, 2 N HCl (4.0 mL) was added, and the solvent was concentrated in vacuo. The residue was diluted with water, and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was recrystallized from EtOAc–hexane to give **62a** (910 mg, 89%) as colorless crystals. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.82–1.95 (2H, m), 2.06–2.13 (2H, m), 2.54–2.62 (1H, m), 3.28–3.37 (2H, m), 3.94–3.98 (2H, m), 7.38–7.44 (3H, m), 8.15–8.19 (2H, m).

5.1.61. 5-(2,5-Diazabicyclo[2.2.1]heptan-2-yl)-3-phenyl-1,2,4-thiadiazole (62b)

To a stirred solution of *tert*-butyl 5-(3-phenyl-1,2,4-thiadiazol-5-yl)-2,5-diazabicyclo[2.2.1]heptane-2-carboxylate (1.16 g, 3.24 mmol) in THF (10 mL) was added 2 N HCl in MeOH (10 mL) dropwise at room temperature. After stirring at room temperature for 12 h, the solvent was concentrated in vacuo. The residue was recrystallized from MeOH–Et_2O to give 5-(2,5-diazabicyclo[2.2.1]heptan-2-yl)-3-phenyl-1,2,4-thiadiazole dihydrochloride as a solid. The solid was dissolved in 1 N NaOH, and extracted with

chloroform. The organic layer was dried over anhydrous $MgSO_4$, and concentrated in vacuo to give **62b** (450 mg, 24%) as a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.56 (1H, s), 1.91 (1H, d, J = 9.9 Hz), 2.00 (1H, d, J = 9.9 Hz), 3.11–3.15 (1H, m), 3.21 (1H, d, J = 10.5 Hz), 3.30 (1H, d, J = 9.9 Hz), 3.67 (1H, d, J = 9.6 Hz), 3.92 (1H, s), 4.58 (1H, br s), 7.41–7.46 (3H, m), 8.17–8.21 (2H, m).

5.1.62. 2-(1,4-Diazepan-1-yl)-4-phenylthiazole (62c)

Compound **62c** was prepared in a manner similar to that described for **62b** in 52% yield as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.94–2.02 (2H, m), 2.93–2.96 (2H, br s), 3.09–3.12 (2H, br s), 3.74 (4H, br s), 7.39–7.43 (3H, m), 8.17–8.20 (2H, m).

5.1.63. 1-(3-Phenyl-1,2,4-thiadiazol-5-yl)piperidin-4-amine (62d)

Compound **62d** was prepared in a manner similar to that described for **62b** in 95% yield as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.28 (2H, br s), 1.43–1.56 (2H, m), 1.93–2.00 (2H, m), 2.95–3.05 (1H, m), 3.21–3.31 (2H, m), 3.97–4.01 (2H, m), 7.39–7.46 (3H, m), 8.16–8.21 (2H, m).

5.1.64. *tert*-Butyl {2-[3-(benzo[*d*]isoxazol-3-yl)ureido]ethyl}carbamate (63)

A mixture of *tert*-butyl (2-aminoethyl)carbamate (20 mg, 0.125 mmol), 2,2,2-trichloroethyl benzo[*d*]isoxazol-3-ylcarbamate (46.4 mg, 0.150 mmol), *N*-ethyldiisopropylamine (0.026 mL, 0.150 mmol), and DMSO (2.0 mL) was stirred at 70 °C overnight, 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 **63** (34.7 mg, 87%) as a colorless powder. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.50 (9H, s), 3.56–3.66 (8H, m), 7.27–7.32 (1H, m), 7.46–7.58 (2H, m), 8.05 (1H, d, J = 8.4 Hz), 8.73 (1H, s).

5.1.65. 1-(2-Aminoethyl)-3-(benzo[*d*]isoxazol-3-yl)urea hydrochloride (64)

To a stirred solution of *tert*-butyl {2-[3-(benzo[*d*]isoxazol-3-yl)ureido]ethyl}carbamate (450 mg, 1.40 mmol) in THF (10 mL) was added 4 N HCl in EtOAc (10 mL) dropwise at room temperature. After stirring at room temperature for 3.0 h, and the solvent was concentrated in vacuo. The residue was recrystallized from MeOH–Et_2O to give **64** (300 mg, 84%) as colorless crystals. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.96 (2H, br s), 3.45–3.51 (2H, m), 7.34–7.39 (1H, m), 7.58–7.66 (3H, m), 7.97 (3H, br s), 8.19 (1H, d, J = 8.1 Hz), 10.36 (1H, s).

5.1.66. *N*-(Benzo[*d*]isoxazol-3-yl)-1-(3-phenyl-1,2,4-thiadiazol-5-yl)piperidine-4-carboxamide (65a)

To a stirred solution of 1-(3-phenyl-1,2,4-thiadiazol-5-yl)piperidine-4-carboxylic acid (289 mg, 1.00 mmol) and DMF (0.010 mL) in THF (5.0 mL) was added oxalyl chloride (0.174 mL, 2.00 mmol) dropwise at 0 °C. After stirring at room temperature for 1 h, the solvent was concentrated in vacuo. To the residue were added THF (5.0 mL), benzo[*d*]isoxazol-3-amine (134 mg, 1.00 mmol), and pyridine (0.404 mL, 5.00 mmol) at 0 °C. The mixture was stirred at room temperature for 1.0 h, and the solvent was concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane–EtOAc), and recrystallized from EtOAc–hexane to give **65a** (60.3 mg, 15%) as colorless crystals, mp 204–205 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.05–2.22 (4H, m), 2.88 (1H, bs), 3.36–3.45 (2H, m), 4.11–4.18 (2H, m), 7.32–7.63 (6H, m), 8.19–8.27 (3H, m), 9.46 (1H, br s). Analytical HPLC showed 97.4% purity.

5.1.67. *N*-(Benzo[*d*]isoxazol-3-yl)-5-(3-phenyl-1,2,4-thiadiazol-5-yl)-2,5-diazabicyclo[2.2.1]heptane-2-carboxamide (65b)

Compound **65b** was prepared in a manner similar to that described for **60c** in 45% yield as colorless crystals, mp 166–167 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.19 (2H, s), 3.66–3.89 (4H, m), 4.92 (1H, br s), 5.04 (1H, s), 7.26–7.31 (1H, m), 7.42–7.56 (5H, m), 8.13–8.22 (4H, m). Anal. Calcd for $C_{21}H_{18}N_6O_2S$: C, 60.27; H, 4.34; N, 20.08. Found: C, 60.13; H, 4.21; N, 20.07.

5.1.68. *N*-(Benzo[*d*]isoxazol-3-yl)-4-(3-phenyl-1,2,4-thiadiazol-5-yl)-1,4-diazepane-1-carboxamide (65c)

Compound **65c** was prepared in a manner similar to that described for **60c** in 54% yield as a white powder, mp 80–81 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.22–2.28 (2H, m), 3.70–3.74 (2H, m), 3.81 (2H, br s), 3.94 (4H, br s), 7.18–7.21 (1H, m), 7.38–7.53 (5H, m), 7.96 (1H, d, J = 8.1 Hz), 8.16–8.19 (2H, m). Analytical HPLC showed 100% purity.

5.1.69. 1-(Benzo[*d*]isoxazol-3-yl)-3-[1-(3-phenyl-1,2,4-thiadiazol-5-yl)piperidin-4-yl]urea (65d)

Compound **65d** was prepared in a manner similar to that described for **60a** in 64% yield as an off-white crystals, mp 264–265 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.62–1.78 (2H, m), 2.04–2.07 (2H, m), 3.41–3.48 (2H, m), 3.93 (3H, br s), 7.34–7.38 (2H, m), 7.47–7.48 (3H, m), 7.64–7.65 (2H, m), 8.10–8.17 (3H, m), 10.10 (1H, s). Analytical HPLC showed 100% purity.

5.1.70. 1-(Benzo[*d*]isoxazol-3-yl)-3-{2-[(3-phenyl-1,2,4-thiadiazol-5-yl)amino]ethyl}urea (65e)

A mixture of 1-(2-aminoethyl)-3-(benzo[*d*]isoxazol-3-yl)urea hydrochloride (200 mg, 0.778 mmol), 5-chloro-3-phenyl-1,2,4-thiadiazole (153 mg, 0.778 mmol), triethylamine (0.433 mL, 3.11 mmol), sodium iodide (117 mg, 0.778 mmol), and MeCN (10 mL) was stirred at 90 °C for 6.0 h, and the solvent was concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc). The product was recrystallized from EtOAc–hexane to give **65e** (69 mg, 23%) as colorless crystals, mp 221–222 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.53 (4H, br s), 7.31–3.43 (4H, m), 7.50 (1H, br s), 7.62–7.64 (2H, m), 8.05–8.08 (2H, m), 8.14(1H, d, J = 8.1 Hz), 8.65 (1H, br s), 10.14 (1H, s). Analytical HPLC showed 99.2% purity.

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, a human brain library was used as a cDNA library, 5′-AAAAGAATTCGCCACCATGGTGCAGTACGAGCTGTG-3′ [SEQ ID NO:1] and 5′-TTTTGTCGACTCAGGATGACTGCTTTT-3′ [SEQ ID NO:2] were used as a primer set, and KOD DNA polymerase (Toyobo Co., Ltd) was used as a DNA polymerase. One cycle of the reaction comprises 95 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, and 45 cycles of the reaction was carried out to obtain an amplified fragment. The amplified fragment was cleaved with restriction enzymes EcoRI and SalI, and then was inserted into a pMSRα vector which had been cleaved with the same restriction enzymes EcoRI and SalI to obtain a pMSRα–human FAAH. A cell line CHO-K1 and the above-obtained plasmid were subjected to a method known per se to prepare the cell line CHO-K1/human FAAH in which human

FAAH was stably expressed. The CHO-K1/human FAAH was cultured in a CO_2 incubator at 37 °C, using a medium (Ham's F-12 medium supplemented with final concentration 10% of fetal bovine serum (FBS) and final concentration 800 μg/mL of G418), 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 900 g, the supernatant was recovered and further centrifuged at 10,000 g. A pellet obtained therefrom was suspended in M-PER (Catalog No. 78501; PIERCE) to give an enzyme fraction.

5.3.2. Enzyme reaction

Using a white walled 96-well plate (Coster Corp.), the test compound at various concentrations, 300 ng of the enzyme fraction and the substrate anandamide [ethanolamine 1-^{3}H] (final concentration 2.5 nM) were reacted in 50 μl of a reaction buffer (125 mM Tris–HCl (pH 9.0), 1 mM EDTA, 0.4 mM HEPES, 0.2% glycerol, 0.02% Triton X-100 and 0.3% fatty acid-free BSA, all at final concentrations) at 37 °C for 30 minutes. The reaction mixture was transferred to a 96-well MultiScreen-HA filter plate (Millipore Corp.) and then was left to stand overnight at room temperature in order to allow the unreacted substrate to be absorbed on the filter. The plate was washed with PBS using a MultiScreen Vacuum Manifold (Millipore Corp.) and dried. To each well, 50 μl of liquid scintillation cocktail was added and stirred, and then counting was performed with a TopCount (Perkin–Elmer Corp.). The count of a sample containing a solvent instead of the test compound was taken as 0%, and the count at zero time was taken as 100%, 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. Compound **60j** was 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. Protein preparation, crystallization and structure analyses

Rat FAAH was prepared as reported previously.[47] In brief, an amino terminal truncation of FAAH (residues Arg30 to Ser579) with an N-terminal hexa-His tag was expressed in *Escherichia coli* Rosetta (DE3). The protein was extracted from cell lysates with 1% Triton X-100 and purified by Ni-NTA agarose (QIAGEN). The eluted protein was further purified by Heparin affinity chromatography using a HiTrap Heparin-Sepharose (GE Healthcare) with 0.05% lauryl-*N*-dimethylaminoxide (LDAO). Eluted fractions were dialyzed against 20 mM HEPES (pH7.5), 150 mM NaCl, 1 mM EDTA, 0.05% LDAO, 5 mM DTT, 10% glycerol. Purified protein was co-concentrated to 5–10 mg/ml with inhibitors using a centrifugal ultrafilter (Amicon Ultra, Millipore).

Crystals were grown utilizing Takeda California's automated nanovolume crystallization technology platform. Crystals suitable for data collection were obtained from sitting-drop vapor diffusion experiments formed from a 50:50 nl mixture of concentrated protein and mother liquor (40% PEG 400, 0.1 M sodium acetate pH 4.5). Crystals were harvested after seven days, flash frozen and stored under liquid nitrogen in an ALS compatible crystal mounting cassette. Diffraction data for the co-crystal structure of FAAH was collected to 2.91 Å from cryo-cooled crystals at the Advanced Light Source (ALS, Berkeley CA) beamline 5.0.2. Data was reduced using the HKL2000 software package[48] in the P3221 space group (a = 103.9 Å, b = 103.9 Å, c = 252.0 Å, alpha = 90.0°, beta = 90.0°, gamma = 120.0°). The structure was determined by molecular replacement with subsequent refinement and model re-building conducted utilizing REFMAC[49] and XtalView[50] software packages. The structures have been deposited with the RCSB structure database with accession codes 4HBP (compound **60d**).

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