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Synthesis and evaluation of *N*-acylsulfonamide and *N*-acylsulfonylurea prodrugs of a prostacyclin receptor agonist

Akio Nakamura, Tetsuhiro Yamada and Tetsuo Asaki*

Discovery Research Laboratories, Nippon Shinyaku Co., Ltd, 14 Nishinosho-Monguchi-Cho, Kisshoin, Minami-Ku, Kyoto 601 8550, Japan

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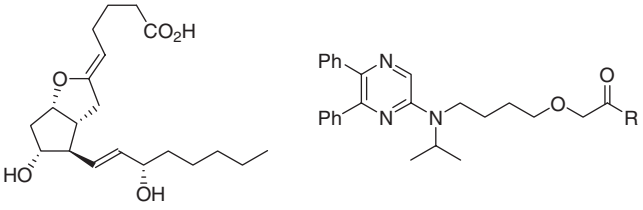
Abstract—*N*-Acylsulfonamide and *N*-acylsulfonylurea derivatives of the carboxylic acid prostacyclin receptor agonist **1** were synthesized and their potential as prodrug forms of the carboxylic acid was evaluated in vitro and in vivo. These compounds were converted to the active compound **1** by hepatic microsomes from rats, dogs, monkeys, and humans, and some of the compounds were shown to yield sustained plasma concentrations of **1** when they were orally administered to monkeys. These types of analogues, including NS-304 (**2a**), are potentially useful prodrugs of **1**.

1. Introduction

Prostacyclin (PGI_2; Fig. 1), an inhibitor of platelet aggregation and potent vasodilator produced by vascular endothelium, is one of the most important regulators of the circulatory system.[1–3] The sodium salt of PGI_2 (Flolan®; GlaxoSmithKline) is used for the therapy of pulmonary arterial hypertension (PAH); its effect is based on its ability to induce vasodilation by activating the prostacyclin receptor (IP receptor).[4] Flolan improves exercise capacity, hemodynamics, and survival in patients with severe PAH.[4] Despite its benefits, however, the PGI_2 therapy has a serious drawback: due to its very short half-life ($t_{1/2}$ = 3–5 min), it requires a complicated delivery system for continuous infusion via a central venous catheter. The most serious complication of this treatment is the incidence of catheter-related sepsis.[5] Therefore, orally available non-prostanoid PGI_2 mimetics with a long duration of action are being sought, and several have been reported.[6–10]

In a previous study,[11] we found the diphenylpyrazine derivative **1** (Fig. 1) to be an orally available IP receptor agonist without the prostanoid skeleton. Compound **1** potently inhibits ADP-induced platelet aggregation in platelet-rich human plasma with an IC_{50} value of 0.2 μM. Furthermore, it has a 130-fold higher affinity (K_i = 12 nM) for the IP receptor than for the seven other human prostanoid receptors (EP_{1-4}, DP, FP, and TP).[12] This compound also showed high bioavailability values (rat, 102%; dog, 80%) and long $t_{1/2}$ values (rat, 3.6 h; dog, 6.2 h; monkey, 5.6 h) in our preclinical studies.[11] In further studies to discover compounds with sustained IP receptor agonist activity, we found that *N*-acylsulfonamide and *N*-acylsulfonylurea derivatives of the carboxylic acid **1** acted as prodrugs of **1**. In this study, various sulfonamide and sulfonylurea derivatives of **1**, including **2a** (NS-304; Fig. 1), were synthesized and their pharmacokinetic characteristics evaluated in vitro and in vivo.



Prostacyclin (PGI_2)

1: R = -OH
2a (NS-304): R = -$NHSO_2Me$

Figure 1. Chemical structures of prostacyclin (PGI_2), **1**, and **2a**.

2. *N*-Acylsulfonamide 2a as a prodrug

The replacement of carboxyl groups by acidic surrogates such as *N*-acylsulfonamide is a useful modification in

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* Corresponding author. Tel.: +81 75 321 9168; fax: +81 75 321 9039; e-mail: t.asaki@po.nippon-shinyaku.co.jp

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drug design,[13–15] because the pK_a value of an acidic NH proton in *N*-acylsulfonamides is similar to that of a carboxylic acid proton.[16] Such modifications often improve the pharmacological characteristics of carboxylic acid drugs.[17,18] We synthesized *N*-acylsulfonamide **2a**, which is derived from **1**, and assayed its potency to inhibit ADP-induced platelet aggregation in human platelet-rich plasma and its binding affinity for the human IP receptor.[12] Unfortunately, this compound showed a 28-fold-reduced potency in the functional assay and a 13-fold lower binding affinity for the human IP receptor compared to the carboxylic acid **1**.[12] However, we have recently found that *N*-acylsulfonamide **2a** is slowly hydrolyzed to the active form **1** by hepatic microsomes from various animals and humans. Little attention has so far been paid to the possibility that *N*-acylsulfonamides are hydrolyzed to the corresponding carboxylic acids in vitro and in vivo. Persico et al.[19] reported that a glycine amide derivative of tolmetin, a non-steroidal anti-inflammatory drug, showed a longer duration of action than did tolmetin itself; this was caused by slow hydrolysis of the prodrug to tolmetin. These findings suggest that *N*-acylsulfonamides as well as the glycine amide derivative of tolmetin can yield sustained plasma concentrations of the active compound if they are hydrolyzed at a suitably slow rate in vivo.

3. In vitro and in vivo evaluation of *N*-acylsulfonamides and *N*-acylsulfonylureas

The hydrolysis of *N*-acylsulfonamide **2a** to **1** was evaluated in plasma and hepatic microsomes from various animals and humans (Table 1). Compound **2a** was hydrolyzed to **1** in the presence of all hepatic microsome preparations tested. Compound **2a** was rapidly hydrolyzed to **1** in the presence of rat plasma, but was not measurably hydrolyzed in the plasma of the other species. These results suggest that **2a** has the potential to be converted to the parent carboxylic acid in vivo in animals and humans.

Esterases play major roles in the hydrolysis of a number of prodrugs in humans and experimental animals. They are mainly classified into three groups, A-, B-, and C-esterases, on the basis of their reactivity with organophosphorus compounds.[20] B-esterases, including carboxylesterase (EC 3.1.1.1.), are serine hydrolases, and carboxylesterase is known to contribute to the hydrolytic biotransformation of many therapeutic agents containing ester or amide bonds.[21] To identify the type of hydrolase responsible for prodrug hydrolysis in the present study, we tested the effect of phenylmethylsulfonyl fluoride (PMSF), a serine hydrolase inhibitor (Table 2). PMSF (100 μM) completely inhibited the hydrolysis of **2a** to **1** in monkey hepatic microsomes, indicating that a serine hydrolase such as carboxylesterase (a B-esterase) was responsible for hydrolysis.

To test whether **2a** is actually converted into **1** in vivo, **2a** was orally administered to monkeys. Compound **1** was detected in the plasma (Table 3), indicating that **2a** had indeed been converted to **1**. The apparent $t_{1/2}$ value for **1** after oral administration of **2a** (10.7 h Table 3) was about twice that observed after administration of **1** itself (5.6 h Table 4). The greater apparent $t_{1/2}$ value for **1** after oral administration of **2a** is probably a result of the slow hydrolysis of **2a** to **1** in monkeys. The C_{max} value for **1** after oral administration of **2a** (35 ng/mL; Table 3) was lower than that observed after oral administration of **1** (105 ng/mL; Table 4). We hypothesize that the typical side effects of IP receptor agonists, such as headache, flush, hypotension, and nausea, are likely to be caused by their excessive vasodilation effects related to a rapid and temporary increase in their plasma concentrations after administration. For this reason, a lower C_{max} value coupled with slow changes in the plasma concentrations of **1** is favorable for a PGI_2 agonist because a relatively high IP receptor agonist activity can be maintained without severe adverse effects. Therefore, the *N*-acylsulfonamide **2a** is considered to be a promising prodrug that is expected to show good pharmacokinetic properties in humans, judging from the similar results obtained for monkeys and humans in in vitro studies, as described above. Indeed, in a microdosing study, **1** was detected in the plasma with a high $t_{1/2}$ value (7.9 h) after oral administration of **2a** to five healthy volunteers.[12]

On the basis of these investigations, we next designed and synthesized analogues of **2a**, the *N*-isopropylsulfonamide **2b**, the trifluoromethanesulfonamide **2c**, the *N*-arylsulfonamides **2d** and **2e**, and the sulfonylureas **2f** and **2g**. The rates of hydrolysis of these derivatives were

Table 1. Hydrolysis of **2a** to **1** in plasma or hepatic microsomes from animals and humans

	Hydrolysis rate (nmol/min/mL or nmol/min/mg protein)			
	Rat	Dog	Monkey	Human
Plasma	0.500	—[a]	—	—
Hepatic microsomes	0.090	0.030	0.057	0.019

[a] Below the limits of detection of **1**.

Table 2. Effect of phenylmethylsulfonyl fluoride on hydrolysis of **2a** to **1** in monkey hepatic microsomes

PMSF (μM)	Hydrolysis rate (nmol/min/mg protein)	% of control
0 (control)	0.0515	100
10	0.0110	21.4
100	0	0
1000	0	0

Table 3. Pharmacokinetic parameters for **2a** and **1** after oral administration of **2a** (1 mg/kg) to monkeys

	2a	**1**
T_{max} (h)	6.7 ± 1.2	14.0 ± 8.7
C_{max} (ng/mL)	47 ± 21	35 ± 8
$t_{1/2}$ (h)	4.9 ± 1.8	10.7 ± 2.2
AUC (ng· h/mL)	384 ± 126	859 ± 152

Each value is the mean ± SD (n = 3).

Table 4. Pharmacokinetic parameters for **1** after oral administration of **1**, the sulfonamides **2a–c**, or the sulfonylurea **2f** (1 mg/kg) to monkeys

Compound administered	Compound measured	*n*	T_{max} (h)	C_{max} (ng/mL)	AUC_{0-24h} (ng·h/mL)	$t_{1/2}$ (h)
1[a]	**1**	3	2.3	105	652	5.6
2a	**1**	3	14.0	35	859	10.7
	2a	3	6.7	47	384	4.9
2b	**1**	2	10	13	170	14.5
	2b	2	10	17	128	2.3
2c[b]	**1**	3	4	31	308	8.5
2f[b]	**1**	3	6	20	374	—[c]

Each value is the mean of two or three animals.
[a] Ref. 11.
[b] The plasma concentrations were not determined because the compound was insufficiently ionized in LC/MS.
[c] Not calculated because the plasma concentrations of **1** after administration of **2f** continued to increase until the end of the sampling period.

Table 5. Hydrolysis of **2a–g** to **1** in hepatic microsomes from monkeys and humans

Compound	Rate of hydrolysis (nmol/min/mg protein)		ClogP
	Monkeys	Humans	
2a	0.057	0.019	4.2
2b	0.152	0.012	5.0
2c	0.060	0.024	6.3
2d	0.711	0.017	5.4
2e	0.468	0.008	5.3
2f	0.112	0.030	3.9
2g	0.208	0.015	5.3

Substrate concentration, 10 μM.
ClogP values were calculated with the program ClogP, version 4.83 (BioByte Corp., Claremont, CA).

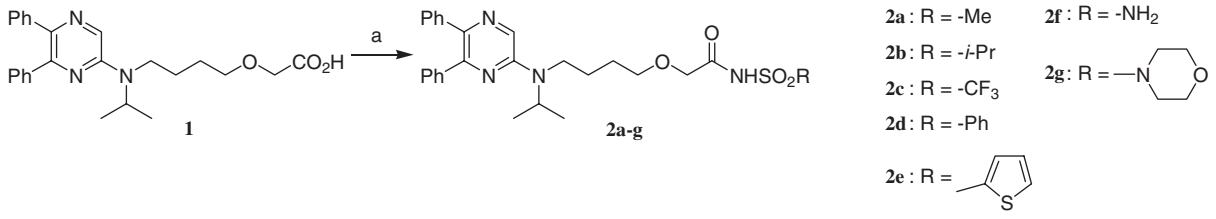
measured in hepatic microsomes from monkeys and humans (Table 5). The rates of hydrolysis of these sulfonamides and sulfonylureas in hepatic microsomes from humans were similar to the rate of hydrolysis of **2a**, so that these compounds, as well as **2a**, are expected to have good pharmacokinetic properties in humans. In contrast, there were large differences in the hydrolysis rates of these compounds in hepatic microsomes from monkeys. In particular, the *N*-arylsulfonamides **2d** and **2e** were converted to **1** more rapidly than the other sulfonamides in this species. Three compounds, **2b**, **2c**, and **2f**, were selected for in vivo studies because their hydrolysis rates were relatively low and similar to the hydrolysis rate of **2a** in monkeys. In pharmacokinetic studies on monkeys, the in vivo conversion of all these compounds to **1** was confirmed in the plasma after oral administration (Table 4). As observed for **2a**, the C_{max} values for **1** after oral administration of the prodrugs were approximately 10–30% lower than the C_{max} value observed after oral administration of **1** itself. In addition, the apparent $t_{1/2}$ values of **1** after oral administration of the prodrugs were 1.5–2.5 times greater than the $t_{1/2}$ value observed after administration of **1** itself. The apparent $t_{1/2}$ values for **1** after oral administration of **2f** could not be calculated because the plasma concentrations of **1** continued to increase until the end of the sampling period. These results demonstrate that various types of sulfonamides and sulfonylureas have the ability to gradually generate the active carboxylic acid in vivo. The plasma concentrations of the active drugs in monkeys are not related simply to the rates of hydrolysis of the prodrugs in monkey microsomes. For example, among the compounds for which pharmacokinetic data are available (Table 4), the rate of hydrolysis of **2b** was the highest (Table 5), yet it yielded the lowest plasma concentrations of **1**. And although **2a** and **2c** show similar rates of hydrolysis, the AUC value for **1** after administration of **2c** was less than half of that observed after administration of **2a**. In general, the plasma concentrations of an active drug after administration of a prodrug depend on many factors in addition to the rate of hydrolysis of the prodrug to the active drug, including the rate of absorption of the prodrug and the presence or absence of metabolism of the prodrug other than hydrolysis to the active drug. The ClogP values of **2a–2g** (Table 5) suggest that these compounds have different liphophilicities and water solubilities. These properties might affect the absorption and oxidative metabolism of the compounds.

4. Synthesis of *N*-acylsulfonamides 2a–e and *N*-acylsulfonylureas 2f and 2g

Compound **1** and *N*-acylsulfonamide **2a** were prepared as previously described.[11] The synthetic methods for compounds **2b–g** are shown in Scheme 1. *N*-Acylsulfonamides **2a–e** and *N*-acylsulfonylureas **2f** and **2g** were easily prepared from the carboxylic acid **1** by condensation with the corresponding sulfonamides and sulfonylureas in the presence of 1,1′-carbonyldiimidazole (CDI) in a manner similar to that described for **2a**. The isopropylsulfonamide[22] and (4-morpholinyl)sulfonamide[23] required for **2b** and **2g**, respectively, were prepared according to the respective literature procedures, and the sulfonamides required for the other compounds were obtained commercially.

5. Conclusions

We have demonstrated that *N*-acylsulfonamides and *N*-acylsulfonylureas derived from the carboxylic acid **1** were converted to **1** in vitro and in vivo, and that some



Scheme 1. Reagents and conditions: (a) i—CDI, THF, rt, 30 min, then reflux, 30 min; ii—RSO_2NH_2, DBU, rt.

of them yielded sustained plasma concentrations of **1** when they were orally administered to monkeys. These types of analogues, including **2a** (NS-304), are therefore thought to have potential as new prodrugs which can exert long-lasting IP receptor agonist activity in humans. Their slow hydrolysis effectively acts as a kind of 'slow-release mechanism' that both prolongs activity and promises to reduce or prevent the adverse effects associated with high concentrations of PGI_2 agonists.

6. Experimental

6.1. Chemistry

Reagents and solvents were used as obtained from the supplier without further purification. Melting points were determined on a Shibata melting-point apparatus, and are uncorrected. Column chromatography was carried out on a silica gel column (Wako Wakogel® C-200). TLC was performed on Merck TLC aluminium sheets silica gel 60 F_{254}, and detection was by UV quenching at 254 nm or spraying with phosphomolybdic acid. Yields were not optimized. ^{1}H NMR spectra were recorded on a Varian Gemini 2000 (200 MHz) spectrometer. Chemical shifts (δ) are given in ppm relative to the internal standard, tetramethylsilane, and coupling constants are given in Hertz (Hz). Mass spectra were recorded on a JEOL JMS-700 mass spectrometer and IR spectra on a Shimadzu FT IR-8100 spectrometer. Compounds **1** and **2a** were prepared in a manner similar to that described previously.[11]

6.1.1. General procedure for the synthesis of 2b–g: 2-{4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}-*N*-(isopropylsulfonyl)acetamide (2b). A mixture of **1** (500 mg, 1.2 mmol) and 1,1′-carbonyldiimidazole (214 mg, 1.3 mmol) in THF (5 mL) was stirred at room temperature for 30 min and at reflux for a further 30 min. After cooling, isopropylsulfonamide[22] (161 mg, 1.3 mmol) was added and the mixture was stirred at room temperature for 10 min. To the mixture 1,8-diazabicyclo[5.4.0]-7-undecene (DBU) (0.18 mL, 1.2 mmol) was added dropwise. After being stirred at room temperature for 36 h, the mixture was poured into 1 N HCl and extracted with EtOAc. The extract was washed with water and dried over $MgSO_4$. After the solvent was concentrated in vacuo, the residue was subjected to chromatography on silica gel with $CHCl_3$/MeOH (70:1) as the eluent. Subsequent recrystallization from EtOAc-*n*-hexane gave **2b** (287 mg, 46%) as pale yellow crystals. Mp 128–129 °C. FAB-MS *m*/*z* 525 $[MH]^+$. IR (KBr): 2938, 1734, 1563, 1485, 1333, 1121, 858, 693 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.29 (6H, d, J = 6.6 Hz), 1.43 (6H, d, J = 7.0 Hz), 1.50–1.90 (4H, m), 3.46 (2H, t, J = 7.2 Hz), 3.59 (2H, t, J = 6.2 Hz), 3.79 (1H, qn, J = 7.0 Hz), 3.97 (2H, s), 4.72 (1H, qn, J = 6.6 Hz), 7.17–7.52 (10H, m), 8.02 (1H, s), 8.50 (1H, br). Anal. Calcd for $C_{28}H_{36}N_4O_4S$: C, 64.10; H, 6.92; N, 10.68. Found: C, 64.19; H, 6.97; N, 10.62.

6.1.2. 2-{4-[(5,6-Diphenylpyrazin-2-yl)(isopropyl)amino]-butoxy}-*N*-(trifluoromethanesulfonyl)acetamide (2c). Compound **2c** was prepared from **1** and trifluoromethanesulfonamide. It was obtained as a pale yellow amorphous powder (54% yield). FAB-MS *m*/*z* 549 $[M-H]^-$. IR (KBr): 2950, 1639, 1559, 1306, 1211, 1184, 698, 631 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.22 (6H, d, J = 6.6 Hz), 1.45–1.80 (4H, m), 3.10–3.70 (4H, m), 3.95 (2H, s), 4.90 (1H, m), 7.10–7.50 (10H, m), 7.89 (1H, s). Anal. Calcd for $C_{26}H_{29}F_3N_4O_4S{\cdot}1.2H_2O$: C, 54.57; H, 5.53; N, 9.79. Found: C, 54.41; H, 5.22; N, 9.45.

6.1.3. 2-{4-[(5,6-Diphenylpyrazin-2-yl)(isopropyl)amino]-butoxy}-*N*-(phenylsulfonyl)acetamide (2d). Compound **2d** was prepared from **1** and phenylsulfonamide. It was obtained as colorless crystals (43% yield). Mp 157–159 °C. FAB-MS *m*/*z* 559 $[MH]^+$. IR (KBr): 3295, 1732, 1559, 1393, 1134, 693, 586 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.30 (6H, d, J = 6.6 Hz), 1.55–1.90 (4H, m), 3.46 (2H, t, J = 7.5 Hz), 3.54 (2H, t, J = 6.3 Hz), 3.84 (2H, s), 4.72 (1H, qn, J = 6.6 Hz), 7.12–7.70 (13H, m), 8.02 (1H, s), 8.08 (2H, d, J = 8.4 Hz). Anal. Calcd for $C_{31}H_{34}N_4O_4S$: C, 66.64; H, 6.13; N, 10.03. Found: C, 66.66; H, 6.12; N, 9.99.

6.1.4. 2-{4-[(5,6-Diphenylpyrazin-2-yl)(isopropyl)amino]-butoxy}-*N*-(2-thienylsulfonyl)acetamide (2e). Compound **2e** was prepared from **1** and thiophene-2-sulfonamide. It was obtained as pale yellow crystals (57% yield). Mp 151–152 °C. FAB-MS *m*/*z* 564 $[MH]^+$. IR (KBr): 2950, 1732, 1561, 1485, 1331, 1120, 695, 579 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.30 (6H, d, J = 6.6 Hz), 1.55–1.90 (4H, m), 3.46 (2H, t, J = 7.0 Hz), 3.55 (2H, t, J = 6.1 Hz), 3.87 (2H, s), 4.71 (1H, qn, J = 6.6 Hz), 7.10 (1H, t, J = 4.1 Hz), 7.15–7.50 (10H, m), 7.67 (1H, d, J = 5.2 Hz), 7.90 (1H, d, J = 3.6 Hz), 8.02 (1H, s). Anal. Calcd for $C_{29}H_{32}N_4O_4S_2$: C, 61.68; H, 5.71; N, 9.92. Found: C, 61.70; H, 5.78; N, 9.76.

6.1.5. *N*-(Aminosulfonyl)-2-{4-[(5,6-diphenylpyrazin-2-yl)-(isopropyl)amino]butoxy}acetamide (2f). Compound **2f** was prepared from **1** and sulfamide. It was obtained as colorless crystals (50% yield). Mp 117–118 °C. FAB-MS *m*/*z* 498 $[MH]^+$. IR (KBr): 3250, 1725, 1561, 1370, 1127, 702 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.26 (6H, d,

J = 6.6 Hz), 1.60–1.95 (4H, m), 3.40 (2H, t, J = 7.9 Hz), 3.62 (2H, t, J = 5.7 Hz), 4.02 (2H, s), 4.95 (1H, qn, J = 6.6 Hz), 6.08 (2H, br), 7.15–7.50 (10 H, m), 7.93 (1H, s). Anal. Calcd for $C_{25}H_{31}N_5O_4S$: C, 60.34; H, 6.28; N, 14.07. Found: C, 60.09; H, 6.27; N, 14.04.

6.1.6. 2-{4-[(5,6-Diphenylpyrazin-2-yl)(isopropyl)amino]-butoxy}-*N*-(4-morpholinylsulfonyl)acetamide (2g). Compound **2g** was prepared from **1** and (4-morpholinyl)sulfonamide.[23] It was obtained as pale yellow crystals (65% yield). Mp 139–140 °C. FAB-MS m/z 568 $[MH]^+$. IR (KBr): 2863, 1725, 1561, 1485, 1161, 1117, 695 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.29 (6H, d, J = 6.6 Hz), 1.50–1.90 (4H, m), 3.38 (4H, t, J = 4.8 Hz), 3.46 (2H, t, J = 7.4 Hz), 3.59 (2H, t, J = 6.0 Hz), 3.73 (4H, t, J = 4.8 Hz), 3.95 (2H, s), 4.74 (1H, qn, J = 6.6 Hz), 7.10–7.50 (10H, m), 8.02 (1H, s), 8.66 (1H, br). Anal. Calcd for $C_{29}H_{37}N_5O_5S$: C, 61.36; H, 6.57; N, 12.34. Found: C, 61.11; H, 6.59; N, 12.03.

6.2. Hydrolysis of 2a to 1 in animal and human plasma

Male Sprague–Dawley rats (Japan SLC, Hamamatsu, Japan), male beagle dogs (Nihon Nosan Kogyo, Yokohama, Japan), and male cynomolgus monkeys (Japan SLC) were used. Human blood was obtained from three healthy non-fasting male volunteers. Blood samples were withdrawn into heparinized test tubes and plasma was prepared by centrifugation.

The assay mixture consisted of plasma (0.45 mL), distilled water (0.045 mL), and compound **2a** (10 μM). After preincubation at 37 °C for 5 min, the reaction was initiated by the addition of 5 μL of a 1 mM solution of **2a** in DMSO. The assay mixture was incubated for 10 min at 37 °C and the reaction was stopped by the addition of 5 mL of EtOAc. The reaction mixture (0.5 mL) was mixed with 0.1 mL of 0.1 N HCl and 25 μL of a methanolic solution of {4-[(5,6-di-*p*-tolylpyrazin-2-yl)(isopropyl)amino]butoxy}acetic acid (10 μg/mL) as an internal standard. Compound **1** was extracted with 5 mL of EtOAc. After centrifugation at 3000 rpm for 10 min, the organic layer was removed and evaporated to dryness and the residue was dissolved in 100 μL of MeOH or acetonitrile. A 20-μL sample of the MeOH or acetonitrile solution was subjected to HPLC on an Inertsil ODS-3 column (4.6 × 150 mm; GL Science, Tokyo, Japan). The mobile phase was 0.1% methanesulfonic acid solution/acetonitrile (5:6). The flow rate was 1 mL/min, the effluent was monitored at 280 nm, and the column was operated at 40 °C.

6.3. Hydrolysis of 2a–g to 1 in hepatic microsomes from various animals and humans

Rat, dog, and monkey hepatic microsomes were purchased from KAC (Kyoto, Japan). The assay mixture consisted of hepatic microsomal protein (0.4 mg/mL), an NADPH-generating system (25 mM glucose-6-phosphate, 4 units/mL glucose-6-phosphate dehydrogenase, 2.5 mM β-$NADP^+$, and 10 mM $MgCl_2$), test compound (10 μM), and 0.25 M phosphate buffer, pH 7.4, in a final volume of 0.5 mL. After a 5-min preincubation at 37 °C, the reaction was started by the addition of 5 μL of test compound solution (1 mM). The assay mixture was incubated for 10 min at 37 °C and the reaction was stopped by the addition of ethanol (100 μL). After centrifugation at 10,000 rpm for 3 min, a 20-μL portion of the supernatant was analyzed by HPLC as described above.

6.4. Identification of the hydrolase type responsible for the hydrolysis of 2a to 1 in monkey hepatic microsomes

The assay mixture consisted of hepatic microsomal protein (1 mg/mL), test compound (**2a**, 10 μM), phenylmethylsulfonyl fluoride (PMSF; 10–1000 μM), and 0.25 M potassium phosphate buffer, pH 7.4, containing 0.25 mM EDTA in a final volume of 0.5 mL. PMSF was dissolved in isopropanol and added in a volume of 5 μL. After a 5-min preincubation at 37 °C, the reaction was started by the addition of 5 μL of a 1 mM solution of the test compound in acetonitrile. The assay mixture was incubated for 10 min at 37 °C and the reaction was stopped by the addition of acetonitrile (500 μL). After centrifugation at 13,000 rpm for 10 min, a 5-μL portion of the supernatant was analyzed by LC/MS/MS as described below.

6.5. Pharmacokinetic study in monkeys

Male cynomolgus monkeys weighing 4–6 kg (Japan SLC) were acclimatized to the laboratory conditions for more than three weeks before dosing at 21–25 °C and 45–65% humidity. The monkeys were fed diet PS (Oriental Yeast Co., Tokyo, Japan). None of the animals were fasted before the experiments. Test compounds were orally administered to two or three animals, and blood samples were collected at various times (before administration and 15 and 30 min and 1, 2, 4, 6, 8, 10, and 24 h after administration) from the saphenous vein. Each blood sample was taken into a heparinized tube and plasma was prepared by centrifugation at 3000 rpm for 15 min at 4 °C. The plasma concentrations of compounds **1**, **2a**, and **2b** after the administration of test compounds were measured by liquid chromatography mass spectrometry (LC/MS) or liquid chromatography tandem mass spectrometry (LC/MS/MS). Briefly, plasma was mixed with an equal volume of 0.2 N HCl and a small amount of methanol solution containing internal standard. Compounds **1**, **2a**, and **2b** were extracted by liquid–liquid extraction or solid-phase extraction. The extracted sample was then assayed by LC/MS/MS. For LC/MS, a Series 1050 liquid chromatograph (Agilent, Palo Alto, CA, USA) was used with an M-2500 magnetic sector-type mass spectrometer (Hitachi, Tokyo, Japan). For LC/MS/MS, an Alliance 2795 liquid chromatograph (Waters, Milford, MA, USA) was used with a Waters Quattro micro API tandem quadrupole mass spectrometer. The ionization mode was atmospheric pressure chemical ionization positive or electrospray ionization positive. The peak intensities of characteristic ions (m/z) were measured in the selected-ion-monitoring mode or the selected-reaction-monitoring mode. HPLC was carried out on a SymmetryShield RP_8 (4.6 × 20 mm, Waters) or Xterra MS_{18}IS (particle size, 3.5 μm; 2.1 × 20 mm, Waters) col-

umn. The mobile phase was acetonitrile/0.1% formic acid (80:20) and the column was operated at a flow rate of 0.3 or 1 mL/min and a temperature of 40 °C.

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