2-{4-[(5,6-Diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}-N-(methylsulfonyl)acetamide (NS-304), an Orally Available and Long-Acting Prostacyclin Receptor Agonist Prodrug

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ABSTRACT

Prostacyclin (PGI₂) and its analogs are useful for the treatment of various vascular disorders, but their half-lives are too short for widespread clinical application. To overcome this drawback, we have synthesized a novel diphenylpyrazine derivative, 2-{4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}-N-(methylsulfonyl)acetamide (NS-304), a prodrug of the active form 2-{4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}acetic acid (MRE-269). NS-304 is an orally available and potent agonist for the PGI₂ receptor (IP receptor). The inhibition constant (Kᵢ) of MRE-269 for the human IP receptor was 20 nM; in contrast, the Kᵢ values for other prostanoid receptors were >2.6 μM. MRE-269 was therefore a highly selective agonist for the IP receptor. The plasma concentrations of MRE-269 remained near peak levels for more than 8 h after oral administration of NS-304 to rats and dogs, and NS-304 increased femoral skin blood flow in rats in a long-lasting manner without affecting the hemodynamics. These findings indicate that NS-304 acts as a long-acting IP receptor agonist in vivo. The continuous vasodilation evoked by NS-304 was not attenuated by repeated treatment, indicating that NS-304 is unlikely to cause severe desensitization of the IP receptor in rats. Moreover, a microdose pharmacokinetic study in which NS-304 was orally administered to healthy male volunteers showed conversion of NS-304 to MRE-269 and a long plasma elimination half-life for MRE-269 (7.9 h). In conclusion, NS-304 is an orally available and long-acting IP receptor agonist prodrug, and its active form, MRE-269, is highly selective for the IP receptor. Therefore, NS-304 is a promising drug candidate for various vascular diseases, especially pulmonary arterial hypertension and arteriosclerosis obliterans.

Five prostanoids, prostaglandin (PG) D₂, PGE₂, PGF₂α, prostacyclin (PGI₂), and thromboxane A₂ (TXA₂), are cyclooxygenase metabolites of arachidonic acid. These lipid mediators interact with specific G protein-coupled receptors (DP, EP₁, EP₂, EP₃, EP₄, FP, IP, and TP) and activate the corresponding intracellular signaling cascade (Narumiya et al., 1992; Breyer et al., 2001).

PGI₂, which is mainly produced in vascular endothelial cells (Moncada et al., 1976; Moncada and Vane, 1978), acts via the PGI₂ receptor (IP receptor) as a potent vasodilator and inhibitor of platelet aggregation (Namba et al., 1994), and it counteracts the actions of TXA₂, a potent vasoconstrictor and initiator of platelet aggregation. Activation of the IP receptor leads to stimulation of adenylate cyclase through coupling to Gₛ protein, with a resulting increase in intracellular cAMP levels (Adie et al., 1992), and to activation of phospholipase C through coupling to Gₛ protein leading to elevation of cytosolic Ca²⁺ concentrations (Vassaux et al., 1992).

The expression of IP receptor mRNA has been observed in various organs, including the heart, lung, thymus, spleen, and kidney (Oida et al., 1995). PGI₂ contributes to the maintenance of homeostasis and various other physiological ef-

ABBREVIATIONS: PG, prostaglandin; PGI₂, prostacyclin; TXA₂, thromboxane A₂; DP, PGD₂ receptor; EP, PGE₂ receptor; FP, PGD₂ receptor; TP, TXA₂ receptor; NS-304, 2-{4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}-N-(methylsulfonyl)acetamide; MRE-269, 2-{4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}acetic acid; CHO, Chinese hamster ovary; h, human; LC/MS, high-performance liquid chromatography coupled to mass spectrometry; r, rat; SQ-29548, [1S-[1α,2α(Z),3α,4α]-7-[3-[[2-[[phenyl]amino]carbonyl]hydrazine]-methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-hep-tonic acid; ONO-1301, [7,8-dihydro-5-[6(E)-2-[[a-(3-pyridyl)benzyldene]amino-oxyl]ethyl]-1-naphthyl-ox] acetic acid; BMY 42393, 2-[3-[[4-(4,5-diphenyl-2-oxazolyl)ethyl]phenoxy] acetic acid; BMY 45778, [3-[[4-(4,5-diphenyl-2-oxazolyl)-5-oxazolyl]phenox] acetic acid.

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fects in many organ systems, for example, the regulation of tissue blood flow, inhibition of thrombus formation, inhibition of the growth of smooth muscle cells, and protection of vascular endothelial cells (Narumiya et al., 1999). Studies on IP receptor-deficient mice have clarified the role of the PGL₃-IP system, and we now know that IP receptor deficiency induces thrombus formation and reduces inflammatory and pain responses (Murata et al., 1997). A decrease in PGI₂ production or an imbalance between PGI₂ and TXA₂ has been reported in several diseases such as pulmonary arterial hypertension (Tuder et al., 1999), ischemic vascular disease (Hirsh et al., 1981), diabetes (Kapen et al., 1982), and glomerulonephritis (Niwa et al., 1987). Treatment with IP receptor agonists or transfer of the PGI₂ synthase gene ameliorates these diseases in animal models (Todaka et al., 1999; Nagaya et al., 2000).

Although beneficial clinical effects of PGI₂ were expected (Armstrong, 1996), PGI₂ itself is too unstable (Moncada and Vane, 1978) for widespread clinical application. For example, although intravenous infusion of PGI₂ can improve exercise tolerance and survival in patients with pulmonary arterial hypertension (Barst et al., 1996), this therapy requires a complicated delivery system for continuous infusion through a central venous catheter because of its very short half-life (3–5 min). A more serious complication of this treatment is the incidence of catheter-related sepsis (Humbert et al., 2004). To overcome these drawbacks, many kinds of PGI₂ analogs have been synthesized, but almost all of them have failed during drug development. Beraprost is the only orally available PGI₂ analog in clinical use for arteriosclerosis obliterans and idiopathic pulmonary arterial hypertension in the Asian region, including Japan. Because the elimination half-life of beraprost is just 1 h (Toda, 1988; Demolis et al., 1993), however, its clinical effects are insufficient even if it is administered three or four times daily. Iloprost is a PGI₂ analog administered by inhalation, and, although it is in clinical use in Europe, frequent dosings are required to maintain its effect.

We have carried out screening for platelet aggregation inhibitors, and we have identified a potent and highly selective nonprostanoid IP receptor agonist, MRE-269. It showed good biological availability and a relatively long half-life compared with established IP receptor agonists on oral administration to animals. Meanwhile, we hypothesized that the typical side effects of IP receptor agonists, such as headache, flush, hypotension or nausea, are probably caused by their excessive vasodilation effects related to a rapid and temporary increase in their plasma concentrations following administration. To improve the pharmacokinetic properties of MRE-269, we sought to reduce the peak-trough fluctuation in the plasma concentrations of MRE-269, and we have synthesized a prodrug of MRE-269, NS-304 (Fig. 1), as a new drug candidate. In animal studies, the elimination half-life of MRE-269 observed after administration of NS-304 was longer than that observed after administration of MRE-269. In addition, the maximal plasma concentrations (C_max) of MRE-269 observed after administration of NS-304 were lower than those observed after administration of MRE-269. In this article, we report the pharmacology and pharmacokinetics of NS-304.

Materials and Methods

Materials. NS-304 (WO 02/088084) and MRE-269 were synthesized in our laboratory. Beraprost was purchased from Chinoin (Budapest, Hungary); [³H]PGD₂ (170 Ci/mm), [³H]PGE₂ (185 Ci/mm), [³H]PGF₂α (184 Ci/mm), and [³H]iloprost (16 Ci/mm) were from GE Healthcare (Little Chalfont, Buckinghamshire, UK); [³H]SQ-29548 (48.2 Ci/mm) was from PerkinElmer Life and Analytical Sciences (Boston, MA); and PGE₂, PGF₂α, PGF₂β, iloprost, and SQ-29548 were from Cayman Chemical (Ann Arbor, MI). A CAMP enzyme-linked immunosorbent assay kit was purchased from GE Healthcare.

Cell Culture. Chinese hamster ovary (CHO) cells (American Type Culture Collection, Manassas, VA) were cultured in F-12 medium containing 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Animals. Male Sprague-Dawley rats and cynomolgus monkeys were purchased from Japan SLC (Hamamatsu, Japan), and male beagle dogs were from Nihon Nosan Kogyo (Yokohama, Japan). Animals were housed in cages under a 12-h light/dark cycle in an animal room maintained at 20–26°C, a relative humidity of 35 to 75%, and a ventilation frequency of at least 12 times/h. Rats were allowed free access to F-2 pellet chow (Funabashi Farm, Funabashi, Japan) and tap water. Dogs and monkeys were given 250 g of TC-1 pellet chow (Aixia Corporation, Tokyo, Japan) and 100 g of PS pellet chow (Oriental Yeast Co., Ltd., Tokyo, Japan) each day, respectively, and they were allowed free access to tap water. All animal procedures were approved by the Committee for the Institutional Care and Use of Animals at Nippon Shinyaku Co., Ltd. (Kyoto, Japan).

Stable Expression of Various Prostanoid Receptors. cDNAs corresponding to the human DP, EP₁, EP₂, EP₃, EP₄, FP, IP, and TP prostanoid receptors and to rat IP receptor were cloned by reverse transcription-polymerase chain reaction. Total RNA was prepared from human kidney or rat spleen. Single-stranded cDNAs were synthesized from human and rat mRNA. Each prostanoid receptor cDNA was inserted into the expression vector pcDNA3.1 (+) with Takara DNA ligation kit, version 2 (Takara, Otsu, Japan) to yield the corresponding prostanoid receptor expression plasmid PGR/pcDNA3.1 (+). The receptor expression plasmid was transfected into CHO cells with Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. After incubation for 48 h, the cells were cultured in F-12 medium containing 600 µg/ml Geneticin (G–418; Invitrogen) for screening of transfection-positive clones. After 3 weeks, colonies of CHO cells were separated by the cloning ring method, and CHO cells expressing the appropriate prostanoid receptor were identified by receptor-binding assays.

Radioligand Binding Assays for Human Prostanoid Receptors and Rat IP Receptor. The binding affinity of each compound for the human prostanoid receptors and the rat IP receptor were

![Figure 1](image-url)
determined by a displacement method as described previously (Kiriyama et al., 1997), with minor modifications. Cultured CHO cells expressing each prostaglandin receptor were harvested and homogenized with 25 mM Tris-HCl buffer, pH 7.4, containing 250 mM sucrose, 10 mM MgCl₂, 1 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 100,000 g for 20 min, and the pellet was homogenized with binding buffer (25 mM Tris-HCl, pH 7.4, containing 10 mM MgCl₂, 1 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride). The homogenate was recentrifuged at 100,000 g for 20 min. The membrane pellet was resuspended in binding buffer. The binding assay was performed in a total volume of 200 μl of binding buffer containing membrane protein (50 μg of TP; 80 μg of EP₂; 100 μg of EP₂, EP₃, EP₄, or TP; or 200 μg of TP) and the corresponding [³H]labeled ligand at 25°C (DP, EP₂, EP₃, EP₄, and TP) for 1 to 2 h. In the saturation binding assay, membrane protein was incubated with various concentrations of [³H]labeled ligands, and, in the displacement binding assay, with [³H]labeled ligand (5 nM [³H]PGF₂α for DP, 5 nM [³H]PGF₂α for EP₂, and 1 nM [³H]PGF₂α for EP₃ and EP₄, 1 nM [³H]PGF₂α for FP, 5 nM [³H]iloprost for IP, and 5 nM [³H]SQ-29548 for TP) in the presence or absence of various concentrations of test compound. Bound ligand was collected by rapid filtration on a glass filter (Whatman GF/B; Whatman, Maidstone, UK). The filter was washed four times with ice-cold 50 mM Tris-HCl buffer, pH 7.4. Radioactivity on the filter was measured with a liquid scintillation counter (Tri-Carb 2700TR; PerkinElmer Life and Analytical Sciences, Boston, MA). The nonspecific binding was defined as the binding in the presence of over 500-fold excess of unlabeled ligand.

The specific binding was calculated by subtraction of the nonspecific binding from the total binding. The dissociation constant (Kᵦ) and maximal number of binding sites (Bₘₐₓ) for the binding of each [³H]labeled ligand to its receptor were calculated. The ligand concentration that gave 50% inhibition (IC₅₀) of specific binding was estimated by linear regression analysis. The inhibition constant (Kᵦ) for each compound was calculated from the equation Kᵦ = IC₅₀/[1 + ([L]/Kᵦ)], where [L] indicates the concentration of [³H]labeled ligand.

Measurement of Intracellular cAMP. CHO cells expressing the human IP receptor (hIP-CHO cells) were seeded at 1 × 10⁶ cells/well in a 24-well plate and cultured for 48 h. The cells were washed with Dulbecco’s phosphate-buffered saline without divalent cations, preincubated in the medium for 1 h at 37°C, and then incubated for 15 min at 37°C with medium containing each drug in the presence of 500 μM 3-isobutyl-1-methylxanthine. The medium was removed, and perchloric acid solution was added to terminate the reaction. Intracellular cAMP levels were measured by enzymelinked immunosorbent assay.

Inhibition of Platelet Aggregation. Each blood sample freshly collected from humans, monkeys, dogs, or rats was mixed with one-tenth volume of 3.8% trisodium citrate solution. Platelet-rich plasma was prepared by centrifugation of the blood samples at 150g for 10 min at room temperature. Platelet aggregation induced by 10 μM ADP was monitored at 37°C with an aggregometer (model PAM-8C; Mebanix, Tokyo, Japan) in the presence or absence of test compound by the method of Born (1962).

Pharmacokinetic Studies in Animals. NS-304 was orally administered to rats at 10 mg/kg and to dogs at 3 mg/kg, and blood samples were collected at various times and centrifuged to obtain plasma. The plasma concentrations of NS-304 and MRE-269 after oral administration of NS-304 to each animal were determined by high performance liquid chromatography coupled to mass spectrometry (LC/MS), and their pharmacokinetic parameters were calculated.

Surgical Procedures in Rats. For femoral skin blood flow (FSBF) and hemodynamics measurements (see below), rats anesthetized with methohexital were maintained at 37°C with a heated pad throughout the experimental period. Pylorus ligation was performed, and the duodenum was exposed and dissected 2 to 3 mm below the pyloric sphincter. A polyethylene tube for administration was inserted into the duodenum and tied in place with suture.

Femoral Skin Blood Flow in Rats. Surgical procedures were performed as described above. A probe for a laser Doppler flowmeter (model ALF21; Advance, Tokyo, Japan) was attached to the femoral skin to monitor the FSBF. After the FSBF had become stable, NS-304, beraprost, or vehicle was administered into the duodenum through the polyethylene tube. The FSBF was recorded for 5 h after intraduodenal administration.

Hemodynamics in Rats. Surgical procedures were performed as described above. A polyethylene tube filled with heparinized saline was inserted into the right femoral artery and connected to a pressure transducer (model DX-360; Nihon Kohden, Tokyo, Japan). The mean arterial blood pressure (MAP) was measured with the pressure transducer via a carrier amplifier (model AP-100F; Nihon Kohden), and the heart rate (HR) was measured with a tachometer (model AT-601G; Nihon Kohden) triggered by blood pressure pulses. After the MAP and HR had become stable, NS-304, beraprost, or vehicle was administered into the duodenum through the polyethylene tube, and the MAP and HR were recorded for 5 h.

Assessment of IP Receptor Desensitization in Rats. Rats were orally administered NS-304 at 3 mg/kg twice daily for 1, 2, 3, or 4 weeks as a pretreatment. On the day after the final administration in the pretreatment, rats were anesthetized with urethane, and the FSBF was measured with a laser Doppler flowmeter after intraduodenal administration of NS-304 at 3 mg/kg as described above.

Microdose Study. An open-label study of the pharmacokinetics, safety, and tolerability of a single microdose of NS-304 in healthy volunteers was conducted in April 2004 in the United Kingdom. The protocol was approved by the local ethics review board, and all volunteers had provided informed consent to take part in the study. The dose was set at 100 μg/subject in accordance with the recommendations in the Committee for Medicinal Products for Human Use Position Paper on Nonclinical Studies to Support Clinical Trials with a Single Microdose (CPMP/SWP/2599/02). After oral administration of NS-304 (100 μg/subject; aqueous solution) to five healthy male volunteers, blood samples were collected at various times and centrifuged to obtain plasma. The plasma concentrations of NS-304 and MRE-269 were determined by high-performance liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS), and their pharmacokinetic parameters were calculated. Safety was assessed by monitoring of vital signs (blood pressure, heart rate, and body temperature), 12-lead electrocardiograms, clinical laboratory tests (hematology, blood chemistry, and urinalysis), physical examination, and reported adverse events.

Statistical Analysis. Statistical significance was evaluated by repeated measure analysis of variance or Dunnett’s test. Differences were considered to be statistically significant at p < 0.05. All statistical analyses were performed with SAS software, version 8.12 (SAS Institute, Cary, NC).

Results

IP Receptor Agonist Activity. [³H]Iloprost showed a single binding affinity for the membranes of both hIP-CHO cells (Kᵦ = 4.07 ± 0.12 nM; Bₘₐₓ = 1245 ± 37 fmol/mg protein; n = 3) and CHO cells expressing the rat IP receptor (rIP-CHO cells) (Kᵦ = 7.95 ± 0.63 nM; Bₘₐₓ = 985 ± 121 fmol/mg protein; n = 3). MRE-269, NS-304, and beraprost inhibited the binding of [³H]Iloprost to the human and rat IP receptors in a concentration-dependent manner (Fig. 2, A and B). Their respective Kᵦ values were 20, 260, and 39 nM for the human IP receptor and 220, 2100, and 19 nM for the rat IP receptor (Table 1). The specific binding to hIP- and rIP-CHO cell membranes is due to the IP receptor, because there was no specific binding of [³H]Iloprost to the mem-
269, NS-304, beraprost, and iloprost, with EC$_{50}$ values of 11.5, 177, 9.4, and 2.0 nM, respectively (Fig. 2C). The intracellular Ca$^{2+}$ levels in hIP-CHO cells were increased by treatment with beraprost or iloprost but not with MRE-269 (data not shown). These findings indicate that MRE-269 is an agonist for the IP receptor with different characteristics from established PGI$_2$ analogs.

**Binding Affinity for Human Prostanoid Receptors.** To determine the receptor selectivity profiles of MRE-269, NS-304, and beraprost, we performed binding assays for various prostanoid receptors. The $K_i$ values of MRE-269 for the DP, EP$_1$, EP$_2$, EP$_3$, FP, and TP receptors were 2.6, >10, 5.8, >10, 4.9, >10, and >10 µM, respectively, and those of NS-304 for all receptors were >10 µM (Table 1). Beraprost bound not only to the human IP receptor but also to the human EP$_3$ receptor, with a $K_i$ value of 680 nM. Beraprost also showed EP$_3$ agonism, with an EC$_{50}$ value of 750 nM (data not shown).

**Inhibition of Platelet Aggregation.** MRE-269 inhibited platelet aggregation in platelet-rich plasma from humans and monkeys at concentrations lower than 10$^{-6}$ M ($IC_{50}$ = 0.21 and 0.21 µM, respectively), whereas concentrations higher than 10$^{-5}$ M were required for the inhibition of aggregation in dogs and rats ($IC_{50}$ = 25 and 10 µM, respectively) (Table 2). NS-304 also inhibited platelet aggregation in humans and monkeys with $IC_{50}$ values of 5.5 and 3.4 µM, respectively, but it showed no inhibition in dogs ($IC_{50}$ of >100 µM). The inhibitory effect of NS-304 on platelet aggregation in rats was not tested because of the rapid hydrolysis of NS-304 to MRE-269 in rat plasma (data not shown). The $IC_{50}$ value of MRE-269 for human platelet aggregation is 26 times lower than that of NS-304. Beraprost also inhibited platelet aggregation with an $IC_{50}$ value on the order of 10$^{-7}$ M in all the species.

**Pharmacokinetic Studies in Animals.** MRE-269 was detected in the plasma of all the species after oral administration of NS-304, indicating that NS-304 had been converted to MRE-269 in vivo (Fig. 3). It was only in rat plasma that NS-304 was not detected, because of the rapid hydrolysis of NS-304 to MRE-269 in rat plasma (data not shown). The plasma concentrations of MRE-269 remained near the peak levels for more than 8 h after oral administration of NS-304 (Fig. 3), and the elimination half-life of MRE-269 after oral administration of NS-304 was 4.4 h in rats and 7.1 h in dogs (Table 3). The $C_{max}$ of MRE-269 after oral administration of NS-304 was 1.1 µg/ml in rats and 9.0 µg/ml in dogs (Table 3).

**Increase in Femoral Skin Blood Flow in Rats.** NS-304 at 1 or 3 mg/kg increased FSBF in anesthetized rats for more than 4 h after intraduodenal administration in a dose-dependent manner (Fig. 4A). In particular, NS-304 at 3 mg/kg caused a sustained increase in FSBF and exhibited a maximal increase of 93% in FSBF 1 h after administration. NS-304 at 0.3 mg/kg had no significant effect. Beraprost at 0.3 mg/kg increased FSBF for approximately 2 h after administration and exhibited a maximal increase of just 43%, but it had no effect at 0.1 or 0.03 mg/kg (Fig. 4B).

**Hemodynamics in Rats.** In anesthetized rats, NS-304 did not decrease the MAP, but it did increase the HR at 3 mg/kg. Although NS-304 at 1 mg/kg significantly increased the FSBF (Fig. 4A), it had no effect on the MAP or HR (Fig. 5A). In contrast, beraprost at 0.3 mg/kg caused changes in the MAP and HR that lasted throughout the observation period.
TABLE 1

<table>
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<tr>
<td>MRE-269</td>
<td>0.020 ± 0.001</td>
<td>&gt;10</td>
<td>5.8</td>
<td>&gt;10</td>
<td>4.9</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>0.22 ± 0.01</td>
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<tr>
<td>NS-304</td>
<td>0.26 ± 0.02</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>2.1 ± 0.1</td>
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<tr>
<td>Beraprost</td>
<td>0.039 ± 0.006</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>0.68</td>
<td>7.2</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>0.019 ± 0.002</td>
</tr>
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</table>

**TABLE 2**

Inhibition of platelet aggregation in platelet-rich plasma

Blood samples were freshly collected into 1/10 volume of 3.8% trisodium citrate solution. Platelet-rich plasma was prepared by centrifugation of blood samples at 150g for 10 min at room temperature. Platelet aggregation induced by 10 μM ADP was monitored at 37°C with an aggregometer in the presence or absence of test compound. Each value is the mean ± S.E.M.; n = 4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Human</th>
<th>Monkey</th>
<th>Dog</th>
<th>Rat</th>
</tr>
</thead>
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<tr>
<td>MRE-269</td>
<td>0.21 ± 0.04</td>
<td>0.21 ± 0.02</td>
<td>25 ± 1.5</td>
<td>10 ± 0.4</td>
</tr>
<tr>
<td>NS-304</td>
<td>5.5 ± 0.8</td>
<td>3.4 ± 1.1</td>
<td>&gt;100</td>
<td>—</td>
</tr>
<tr>
<td>Beraprost</td>
<td>0.019 ± 0.002</td>
<td>0.025 ± 0.001</td>
<td>0.035 ± 0.004</td>
<td>0.037 ± 0.001</td>
</tr>
</tbody>
</table>

—, not tested.

period, bringing about a maximal decrease of 26% in MAP and a maximal increase of 47% in HR (Fig. 5B).

**Assessment of IP Receptor Desensitization in Rats.** To assess whether the effects of NS-304 on FSBF are attenuated by repeated administration, we tested rats that had been pretreated with NS-304 at 3 mg/kg twice daily for 1, 2, 3, or 4 weeks. Intraduodenal administration of NS-304 at 3 mg/kg to pretreated rats markedly increased the FSBF to about the same extent as rats that were not pretreated, irrespective of the length of the pretreatment period (Fig. 6). There were no significant differences among the groups in intraduodenally administered NS-304 by repeated measures analysis of variance.

**Microdose Study.** To test the pharmacokinetic profiles of NS-304 and MRE-269 in humans, a microdose study was conducted in which 100 μg of NS-304 was orally administered to five healthy male Caucasian volunteers (mean age, 23.8 years; range, 21–30 years). After a rapid peak of NS-304, MRE-269 was detected in the plasma 15 min after administration (Fig. 7). Twelve hours after administration, the plasma concentrations of MRE-269 remained at approximately 17% of the Cₘₐₓ value, and the elimination half-life of MRE-269 was 7.9 h (Table 4). Five adverse events were reported by four subjects, including two headaches. All adverse events were considered to be mild in intensity, and only one (headache) was considered to be possibly related to studying the drug by the investigator. There were no physiologically significant changes in blood pressure, heart rate, or body temperature, nor were physiologically significant changes revealed by electrocardiogram, clinical laboratory tests, or physical examination (data not shown).

**Discussion**

To overcome the drawbacks of PGI₂ itself and its analogs related to their short half-lives, we synthesized a novel diphenylpyrazine derivative, NS-304, a prodrug of the active form MRE-269 (Fig. 1).

MRE-269 showed a high binding affinity for the human IP receptor, with a Kᵢ value of 20 nM, so that it is as potent as various PGI₂ analogs such as beraprost (Kᵢ = 39 nM in the present study), iloprost (Kᵢ = 11 nM), and cicaprost (Kᵢ = 17 nM) (Abramovitz et al., 2000). The intracellular cAMP levels in hIP-CHO cells were increased in a concentration-dependent manner by treatment with MRE-269, which also inhibited platelet aggregation in various species. These findings show that MRE-269 is a potent IP receptor agonist.

The binding affinity of MRE-269 for the human IP receptor was >130 times higher than its affinity for other human prostanoid receptors. MRE-269 is therefore a highly selective IP receptor agonist. NS-304 also had very weak affinity for prostanoid receptors besides the IP receptor, with Kᵢ values of >10 μM. In contrast, most PGI₂ analogs show poor selectivity for the IP receptor because they also have high affinity for the EP subtypes. In the present study, beraprost bound not only to the human IP receptor but also to the human EP₃ receptor, with a Kᵢ value of 680 nM. This is in good agreement with the binding affinity profile of beraprost reported for the mouse prostanoïd receptors (Kᵢ for IP = 16 nM; Kᵢ for EP₃ = 110 nM) (Kiriyama et al., 1997). Iloprost binds to the human IP, EP₁, and EP₃ receptors, with Kᵢ values of 11, 15, and 56 nM, respectively, and cicaprost binds to the human IP, EP₃, and EP₄ receptors, with Kᵢ values of 17, 255, and 44 nM, respectively (Abramovitz et al., 2000). Furthermore, a nonprostanoid IP receptor agonist ONO-1301 also binds to the mouse EP₃ receptor (Kᵢ for IP = 47 nM; Kᵢ for EP₃ = 740 nM) (Kiriyama et al., 1997). Therefore, MRE-269 has a novel and favorable selectivity profile for prostanoid receptors.

The IC₅₀ of MRE-269 for platelet aggregation in humans and monkeys is much lower than it is in dogs and rats. Likewise, a nonprostanoid IP receptor agonist, octimibate, is a potent inhibitor of platelet aggregation in humans and monkeys, but it is less potent in other species (Merritt et al., 1991). Species differences were also observed in the binding affinity of MRE-269 to the IP receptor, and the Kᵢ value of MRE-269 for the human IP receptor was less than one-tenth that of MRE-269 for the rat IP receptor.
In pharmacokinetic studies in animals, MRE-269 was detected in the plasma of each species after oral administration of NS-304, indicating that NS-304 had been converted to MRE-269 in vivo. The plasma concentrations of MRE-269 were maintained near the peak level for more than 8 h after oral administration of NS-304. The elimination half-life of MRE-269 after oral administration of NS-304 was 4.4 h in rats and 7.1 h in dogs. Therefore, orally administered NS-304 is converted into MRE-269, which then evokes highly selective IP receptor agonism in a long-lasting manner.

**Table 3**
Pharmacokinetic parameters of NS-304 and MRE-269 after oral administration of NS-304 to rats and dogs

<table>
<thead>
<tr>
<th>Species</th>
<th>Compound</th>
<th>$T_{\text{max}}$ (h)</th>
<th>$C_{\text{max}}$ (ng/ml)</th>
<th>$t_{1/2}$ (h)</th>
<th>Area Under the Curve (ng·h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>MRE-269</td>
<td>1.3 ± 0.6</td>
<td>1.1 ± 0.1</td>
<td>4.4 ± 1.4</td>
<td>11.6 ± 0.7</td>
</tr>
<tr>
<td>Dog</td>
<td>NS-304</td>
<td>0.8 ± 0.3</td>
<td>7.6 ± 3.1</td>
<td>1.8 ± 0.5</td>
<td>15.0 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>MRE-269</td>
<td>3.3 ± 0.6</td>
<td>9.0 ± 0.8</td>
<td>7.1 ± 1.5</td>
<td>116 ± 21</td>
</tr>
</tbody>
</table>

**Fig. 3.** Plasma concentrations of NS-304 and MRE-269 after oral administration of NS-304 to rats (A) and dogs (B). After NS-304 was orally administered to rats at 10 mg/kg and dogs at 3 mg/kg, the plasma concentrations of NS-304 and MRE-269 were determined by LC/MS at each time point. Each point represents the mean ± S.D.; $n = 3$.

**Fig. 4.** Effects of NS-304 (A) and beraprost (B) on FSBF in rats. NS-304, beraprost, or vehicle was intraduodenally administered to anesthetized rats, and the FSBF was monitored with a laser Doppler flowmeter for 5 h after administration. Each point represents the mean ± S.E.M.; $n = 8$. *, $p < 0.05$; **, $p < 0.01$ versus zero time by Dunnett’s test.

PGL₄ and its analogs cause an increase in skin blood flow (Nishio et al., 1989; Ueno et al., 1996). To assess the efficacy of NS-304 action via IP receptor agonism in vivo, FSBF was measured in anesthetized rats after intraduodenal administration of NS-304. Consistent with the pharmacokinetic profile of MRE-269 after oral administration of NS-304, intraduodenal administration of NS-304 increased FSBF in a long-lasting and dose-dependent manner. In particular, NS-304 at 1 mg/kg caused a sustained increase in FSBF without affecting the MAP and HR. In contrast, although beraprost at the highest dose of 0.3 mg/kg increased FSBF for approximately 2 h, it severely decreased the MAP and increased the...
This result indicates that 0.3 mg/kg is likely to be the maximal tolerable dose of beraprost in rats. Moreover, NS-304 yielded a maximal increase in FSBF of 93% at 3 mg/kg, whereas beraprost yielded a maximal increase of just 43%, even at the highest dose. Therefore, the pharmacological and pharmacokinetic characteristics of NS-304 were clearly more favorable than those of beraprost. Specifically, NS-304 was superior to beraprost in its efficacy, persistence in the plasma, and safety margin with respect to hemodynamic effects. It is possible that the lower efficacy of beraprost in increasing FSBF is related to its poor selectivity for the IP receptor, because beraprost also showed EP3 agonism (EC50 = 750 nM in the present study). EP3 receptor agonists cause arterial contraction (Qian et al., 1994; Jones et al., 1998), and the presence of a sensitive EP3 contractile system interferes with IP receptor-mediated vasorelaxation (Jones et al., 1997; Chan and Jones, 2004). Therefore, the effect of beraprost on the increase in FSBF is likely to have been attenuated by EP3 receptor agonism. In addition, the pharmacological profiles of nonprostanoid PGI2 agonists (octimibate, BMY 42393, and BMY 45778) and PGI2 analogs (cicaprost, iloprost, and carbacyclin) are different in the activation of signal transduction, such as the phospholipase C pathway (Chow et al., 2001). The nonprostanoid PGI2 agonist MRE-269 did not activate Ca2+ signaling, whereas the PGI2 analog beraprost and iloprost did. The difference in pharmacological characteristics between MRE-269 and beraprost may be attributable to this difference in the activation of signal transduction. Moreover, beraprost has a positive chronotropic effect on isolated guinea pig atria (Ueno et al., 1996), which suggests that the potent hemodynamic effects of beraprost are partially due to its direct effect in increasing the heart rate.

Long-term exposure of the IP receptor to PGI2 analogs in vitro causes rapid and severe desensitization of the receptor (Nilius et al., 2000; Smyth et al., 2000; Sobolewski et al., 2004). In addition, dose escalation is required to maintain efficacy in continuous infusion therapy with PGI2 in patients with pulmonary arterial hypertension. However, the amount of desensitization is likely to have been overestimated in the in vitro studies because drastic desensitization was observed several hours after stimulation with PGI2 analogs, whereas dose escalation of PGI2 in the clinical situation is not very frequent. The overestimates of desensitization in those studies are probably due to

**Fig. 5.** Time course of changes in MAP (A) and HR (B) in rats. NS-304, beraprost, or vehicle was intraduodenally administered to anesthetized rats. The MAP was monitored with a pressure transducer via a carrier amplifier, and the HR was monitored with a tachometer triggered by blood pressure pulses. Each point represents the mean ± S.E.M.; n = 4. *, p < 0.05; **, p < 0.01 versus zero time by Dunnett’s test.

**Fig. 6.** Effect of NS-304 on FSBF in rats pretreated with NS-304 for up to 4 weeks. Rats were orally administered NS-304 at 3 mg/kg twice daily for 1 to 4 weeks as a pretreatment. The pretreated rats were anesthetized, and the FSBF was monitored with a laser Doppler flowmeter for 5 h after intraduodenal administration of NS-304 at 3 mg/kg. Each point represents the mean ± S.E.M.; n = 5 (pretreatment 2 w, n = 4).

HR. This result indicates that 0.3 mg/kg is likely to be the maximal tolerable dose of beraprost in rats. Moreover, NS-304 yielded a maximal increase in FSBF of 93% at 3 mg/kg,
the practice of taking a decrease in cAMP production as a measure of IP receptor desensitization. For example, the maximal effect of various PGL₂ analogs on the inhibition of cell proliferation is about the same even when the maximal increases in cAMP evoked by various PGL₂ analogs are markedly different (Clapp et al., 2002). This result suggests that the increase in cAMP production induced by an IP receptor agonist does not necessarily reflect its efficacy, and, conversely, a decrease in cAMP production does not necessarily reflect an attenuation of efficacy. To clarify the effect of repeated administration of NS-304 on its efficacy, we assessed IP receptor desensitization in rats pretreated with NS-304 at 3 mg/kg twice daily for 1 to 4 weeks. The pretreatment dose was 3 times higher than the minimal dose at which NS-304 increased the FSBF in rats. In the plasma concentrations of MRE-269 were 45% of Cₘₐₓ 10 h after oral administration of NS-304. Therefore, effective plasma concentrations of MRE-269 were probably maintained all day long by the pretreatment. That intraduodenal administration of NS-304 at 3 mg/kg to pretreated rats markedly increased the FSBF in a long-lasting manner just as in rats that were not pretreated, and irrespective of the length of the pretreatment period, indicates that there was no significant attenuation of the effect of NS-304 after repeated administration. These findings strongly suggest that NS-304 does not cause rapid or severe desensitization of the IP receptor.

A microdose pharmacokinetic study in humans showed conversion of NS-304 to MRE-269 and a long plasma elimination half-life for MRE-269 (7.9 h), approximately 8 times longer than that of beraprost (Toda, 1988; Demolis et al., 1993). Continuous infusion of PGL₂ via a central venous catheter can improve exercise tolerance and survival in patients with pulmonary arterial hypertension (Barst et al., 1996), but this therapy causes serious complications, such as sepsis, and it reduces the quality of life (Humbert et al., 2004). NS-304, which brings the excellent pharmacokinetics of MRE-269, can overcome these problems resulting from the short half-life of PGL₂.

In conclusion, NS-304 is an orally available and long-acting IP receptor agonist prodrug, and its active form, MRE-269, is highly selective for the IP receptor. Therefore, it is a promising drug candidate for various vascular diseases, especially pulmonary arterial hypertension and arteriosclerotic obliterans.

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References


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