Pharmacological Profile of GPD-1116, an Inhibitor of Phosphodiesterase 4

Takashi Nose, Miwa Kondo, Masashi Shimizu, Hiroki Hamura, Yusuke Yamaguchi, Takako Sekine, and Kouki Ishitani


Received August 24, 2015; accepted January 25, 2016

We have previously reported that GPD-1116, an inhibitor of phosphodiesterase (PDE) 4, exhibits anti-inflammatory effects in a model of cigarette smoke-induced emphysema in senescence-accelerated P1 mice. In the present study, we further characterized the pharmacological profile of GPD-1116 in several experiments in vitro and in vivo. GPD-1116 and its metabolite GPD-1133 predominantly inhibited not only human PDE4, but also human PDE1 in vitro. Moreover, GPD-1116 was effective in several disease models in animals, including acute lung injury, chronic obstructive pulmonary disease (COPD), asthma and pulmonary hypertension; the effective doses of GPD-1116 were estimated to be 0.3–2 mg/kg in these models. With regard to undesirable effects known as class effects of PDE4 inhibitors, GPD-1116 showed suppression of gastric emptying in rats and induction of emesis in dogs, but showed no such suppression of rectal temperature in rats, and these side effects of GPD-1116 seemed to be less potent than those of roflumilast. These results suggested that GPD-1116 could be a promising therapeutic agent for the treatment of inflammatory pulmonary diseases. Furthermore, the inhibitory effects of GPD-1116 for PDE1 might be associated with its excellent pharmacological profile. However, the mechanisms through which PDE1 inhibition contributes to these effects should be determined in future studies.

Key words phosphodiesterase; naphthyridine; pulmonary inflammation

Enzymes of the cyclic nucleotide phosphodiesterase (PDE) family hydrolyze both cAMP and cGMP. PDE isozymes have been grouped into 11 classes according to their substrate specificity and biochemical features. Because inhibition of PDE activity increases intracellular concentrations of cyclic nucleotides by suppressing these degradation to 5'-nucleotides and alters intracellular signal transduction, the PDE family has been recognized as promising targets for therapeutic drugs. For example, inhibitors of PDE3 and PDE5 have actually been used in medicinal therapy for acute heart failure, erectile dysfunction and pulmonary hypertension. Furthermore, inhibition of PDE4 results in excellent anti-inflammatory effects under experimental conditions, and an inhibitor of PDE4, roflumilast, has recently emerged as a therapeutic drug for the treatment of chronic obstructive pulmonary disease (COPD).

We have previously reported that GPD-1116, a novel PDE4 inhibitor created by ASKA Pharmaceutical, can attenuate the inhibitory effects of PDE4 in several experiments in vitro and in vivo.

MATERIALS AND METHODS

Animals Male CD Sprague-Dawley (SD) rats, male Hartley guinea pigs and female Beagle dogs were obtained from Charles River Laboratories Japan (Kanagawa, Japan), Kyudo (Saga, Japan)/Japan SLC (Shizuoka, Japan) and Yakken Farm (Hyogo, Japan), respectively. The animals were housed in temperature-controlled rooms at 20–26°C with a 12-h light-dark cycle. Rats and guinea pigs were allowed free access to water and fed with 300 g of standard feed daily. Rats, guinea pigs and dogs were used at 6 weeks of age, 5–6 weeks of age and 1 year of age, respectively, at the initiation of each experiment. All procedures were approved by the Animal Research Committee of ASKA Pharmaceutical.

Test Compounds and Reference Compounds GPD-1116 (3-benzyl-5-phenyl-1H-pyrazolo[4,3-c][1,8]naphthyridin-4(5H)-one), and GPD-1133 (3-(4-hydroxybenzyl)-5-phenyl-1H-pyrazolo[4,3-c][1,8]naphthyridin-4(5H)-one), a main metabolite of GPD-1116 found in rats, guinea pigs and humans, were used as test compounds (Fig. 1). Roflumilast (a PDE4 inhibitor), montelukast (a leukotriene blocker), rolipram (a PDE4 inhibitor) and tadalafil (a PDE5 inhibitor) were used as reference compounds. Roflumilast and tadalafil were synthesized by ASKA Pharmaceutical, and montelukast and rolipram were purchased from LKT Laboratories and Sigma-Aldrich (U.S.A.), respectively. For in vitro experiments, the compounds were dissolved in dimethyl sulfoxide (DMSO),
GPD-1133 is the main monohydroxy metabolite of GPD-1116 found in rats, guinea pigs, and humans.

whereas for in vivo experiments, the compounds were suspended in 0.5% sodium carboxymethylcellulose.

**PDE Enzymes** Recombinant human PDE isozymes were purchased from Scottish Biomedical (U.K.) or Calbiochem (U.S.A.). PDE4 from U937 cell lysates was purified in our laboratory using ion-exchange chromatography according to previous studies.8–10

**Reagents** [³H]-cAMP and [³H]-cGMP were purchased from PerkinElmer, Inc. or GE Healthcare (U.S.A.). Lipopolysaccharide (LPS; from E. coli, serotype 055:B5), ovalbumin (OVA), monocrotaline (MCT), 5′-nucleotidase, calmodulin, pyrilamine maleate salt and bovine serum albumin (BSA) were obtained from Sigma-Aldrich. Gelatin zymogram gels were purchased from Invitrogen. Other reagents of ultra-grade were used in all experiments.

**Inhibition of PDE Isozymes in Vitro** Inhibition of PDE isozymes by test and reference compounds was measured using a radioisotopic method.8 Briefly, test compounds and roflumilast were added to 50 mM Tris–HCl buffer containing [³H]-cAMP (1 μM) or [³H]-cGMP (1 μM), 5′-nucleotidase (4 μg/mL), 6 mM MgCl₂, 2.5 mM dithiothreitol (DTT), 0.23 mM BSA. In case of PDE1 activity, calmodulin (20 units/mL) and BSA. In case of PDE1 activity, calmodulin (20 units/mL) and CaCl₂ (0.1 mM) were also added to the reaction buffer. Then, each PDE isozyme (1 unit/tube) was added to commence the reaction, and the mixtures were incubated for 20 min at 30°C. The reactions were terminated by the addition of 1 mL of 50% (w/v) AG1-X8 resin slurry (Bio-Rad Laboratories, U.S.A.) and CaCl₂ (0.1 mM) were also added to the reaction buffer. Then, each PDE isozyme (1 unit/tube) was added to commence the reaction, and the mixtures were incubated for 20 min at 30°C. The reactions were terminated by the addition of 1 mL of 50% (w/v) AG1-X8 resin slurry (Bio-Rad Laboratories, U.S.A.) and each supernatant of the reaction mixture was then dissolved in 5 mL of scintillation cocktail. The radioactivity was measured using a liquid scintillation counter.

**Cigarette Smoke Extract (CSE)+LPS-Induced COPD Model in Guinea Pigs** A CSE plus LPS-induced COPD model in guinea pigs was established as described previously.13 Briefly, 200 μL of CSE, which was made by bubbling the smoke of one cigarette (Hi Lite, Japan Tabaco) in 1 mL of saline, was intratracheally instilled into guinea pigs once daily on days 1–4, 6–9, 11–14, and 16–19, and 200 μL of LPS solution (500 μg/mL) was intratracheally instilled into the guinea pigs once daily on days 5, 10, and 15. On day 20, the guinea pigs were anesthetized with sodium pentobarbital (35 mg/kg, i.p.), and lungs isolated were fixed with 10% neutral-buffered formalin solution under a pressure of 25 cm H₂O for 4 h and then soaked for more than 24 h. Sections of the lungs were stained with Hematoxylin-Eosin, and histopathological changes, were evaluated under a microscope.7,13 In order to evaluate the change in air space, mean linear intercept (MLI) was measured as an index according to the previous report.7 GPD-1116 (1 mg/kg) was orally administered to guinea pigs once daily 1 h before CSE or LPS instillation on days 1–19.

**Antigen-Induced Biphasic Asthma Model in Guinea Pigs** Guinea pigs were exposed to aerosolized 1% OVA/saline for 10 min once daily for 8 consecutive days. One week after the final sensitization, antigen challenge was performed by exposure to aerosolized 2% OVA/saline for 5 min, and then the guinea pigs were immediately placed into chambers. Specific airway resistance (sRaw) was measured in each guinea pig using a two-chambered, double-flow plethysmograph system (Plumos-1; M.I.P.S.) and analyzed using WinPUL13 software. As indexes for evaluation of GPD-1116 and montelukast, the increase rates in sRaw at 1 min after antigen challenge (immediate asthmatic response (IAR)) and the area under curve (AUC) of the increase in sRaw between 4 and 8 h after antigen challenge (late asthmatic response (LAR)) were used. GPD-1116 (0.03–3 mg/kg) and montelukast (3 mg/kg) were orally administered to guinea pigs before antigen challenge. In order to enhance LAR and avoid anaphylactic shock, the guinea pigs were treated with metyrapone (10 mg/kg, intravenously) 1 and 24 h before antigen challenge and with pyrilamine maleate (10 mg/kg, i.p.) 30 min before antigen challenge.

**Antigen-Induced Lung Eosinophilia Model in Guinea Pigs** Antigen sensitization and antigen challenge to guinea pigs were performed as described above. Twenty-four hours after antigen challenge, the guinea pigs were anesthetized with sodium pentobarbital (100–200 mg/kg, i.p.), and BAL was performed twice with 5 mL of saline. The number of leukocytes in BALF was counted, and cell population was identi-
fied under a microscope by May–Grünwald Giemsa staining. GPD-1116 (0.01–1 mg/kg) was orally administered to guinea pigs 1 h before antigen challenge.

MCT-Induced Pulmonary Hypertension Model in Rats

MCT-induced pulmonary hypertension was induced by treatment with MCT (40 mg/kg, subcutaneously) according to standard protocols. Four weeks after MCT treatment, systemic arterial pressure (SAP) and right ventricular systolic pressure (RVSP) were measured. Measurements of SAP and RVSP were initiated when stable pressures were recorded for more than 5 min. After the measurements, the heart was isolated, and the ratio of the right ventricle weight to the left ventricle weight+septum weight [RV/(LV+S)] was measured as an index of right ventricular hypertrophy. GPD-1116 (2 mg/kg), roflumilast (2 mg/kg) and tadalafil (10 mg/kg) were orally administered to rats beginning on day 1, 5 times/week, for 4 weeks.

Pulmonary cAMP Levels in Rats

Influence of GPD-1116, roflumilast and tadalafil on pulmonary cAMP levels was investigated in rats as described previously. Briefly, rats were orally treated with GPD-1116 (2 mg/kg), roflumilast (2 mg/kg) or tadalafil (10 mg/kg) for 7 consecutive days, and then the rats were sacrificed by exsanguination under anesthesia with isoflurane 6 h after the final administration. Lungs isolated were fixed and homogenized in 10-fold volume of 5% trichloroacetic acid on ice. The concentrations of cAMP in the supernatants of the homogenates were measured with BCA protein assay kits (Pierce, U.S.A.) in order to correct for the variance of the protein concentrations of the pellet were measured with BCA protein assay kits (Pierce, U.S.A.) in order to correct for the variance of the procedure.

Rectal Temperature in Rats

GPD-1116 (3 mg/kg) or roflumilast (3 mg/kg) was orally administered to rats fasted for 24 h. One hour after the administration, rectal temperature was recorded with a thermistor type thermometer (1-A5388, Natume Seisakusho, Japan) just prior to drug administration and 15, 30, 60, 120, and 180 min after administration.

Emesis in Dogs

GPD-1116 (0.5–3 mg/kg), roflumilast (0.5 and 1 mg/kg) or rolipram (0.5 and 1 mg/kg) was orally administered to dogs 1 h after the feeding with 300 g of special feed (Pedigree™, MARS Japan). Vomiting behavior was observed for 4 h after the administration.

Data Analysis

F tests, Student’s t-tests, Welch’s t-tests, Bartlett’s tests, Dunnett’s parametric multiple comparison tests and Steel’s tests were performed. In the CSE+LPS-induced COPD model, Wilcoxon’s rank sum tests were performed between the negative control and CSE+LPS control groups or between the CSE+LPS control and GPD-1116 treatment groups.

All tests were conducted as one-sided tests using StatLight Ver. 2000C (Yukms Co., Ltd., Japan) or SAS Stat Preclinical Ver. 1.1.3631 (SAS Institute Inc., U.S.A.), and the differences were regarded as significant when the hazard ratio was less than 5%.

### RESULTS

#### Inhibition of PDE Isozymes by GPD-1116 and GPD-1133 in Vitro

In preliminary experiments with crude enzyme preparations from porcine and bovine tissues, GPD-1116 and GPD-1133, a main metabolite of GPD-1116 in rats, guinea pigs and humans, predominantly inhibited both PDE4 and PDE1 among PDE1–6 (data not shown). Thus, we focused on analyzing the inhibitory effects of GPD-1116 and GPD-1133 on human PDE1 and PDE4 subtypes using recombinant enzymes in this study. Additionally, the inhibition of human PDE7–11 by GPD-1116 and GPD-1133 was also evaluated because of the lack of data in porcine and bovine preparations.

As shown in Table 1, GPD-1116 broadly inhibited each subtype of PDE1 and PDE4; in particular, inhibition of PDE1A, PDE1C, PDE4A, PDE4B and PDE4D was slightly stronger than inhibition of other subtypes of PDE1 and PDE4. The IC50 values of GPD-1116 for PDE1A3, PDE1B1 and PDE1C1 were 0.032, 0.79 and 0.025 µM, respectively, and those for PDE4A4, PDE4B2, PDE4C2 and PDE4D3 were 0.10, 0.20, 0.063 and 0.050 µM, respectively. GPD-1133 was slightly more potent at inhibiting PDE1 and PDE4 subtypes than GPD-1116; however, the tendency for inhibition of PDE1 and PDE4 subtypes was similar. The IC50 values of GPD-1133 for PDE1A3, PDE1B1 and PDE1C1 were 0.025, 0.25 and 0.025 µM, respectively, and those for PDE4A4, PDE4B2, PDE4C2 and PDE4D3 were 0.040, 0.20, 0.063 and 0.050 µM, respectively. Both GPD-1116 and GPD-1133 did not inhibit PDE7, PDE8, PDE9 or PDE11, but slightly inhibited PDE10. In contrast, roflumilast showed strong and specific inhibition of PDE4 subtypes, and the IC50 values of roflumilast for PDE4A4, PDE4B2, PDE4C2 and PDE4D3 were 0.00017, 0.000027, 0.0015 and 0.000015 µM, respectively.

#### Inhibitory Effects of GPD-1116 on LPS-Induced Lung Neutrophilia and MMP-9 Induction in Rats

Next, we compared the effects of GPD-1116 and roflumilast on LPS-induced acute lung inflammation in rats. As shown in Fig. 2A, LPS induced a marked increase in neutrophils in the BALF;
the mean number of neutrophils was $70 \times 10^5$ cells/rat in the LPS control group, and few neutrophils were found in the BALF from rats in the negative control group. GPD-1116 and roflumilast significantly and dose-dependently inhibited the infiltration of neutrophils into the airway, with ED$_{50}$ values of 0.18 and 0.70 mg/kg, respectively. The effective dose of roflumilast in the present study was comparable with those described in previous reports. In this model, increase in gelatinolytic activity in BALF was also observed in the LPS control group as a main band at 92 kDa (proMMP-9) on gelatin zymogram gels (Fig. 2B). Both GPD-1116 and roflumilast potently attenuated the gelatinolytic activities of MMP-9 in BALF at doses of 0.5–2 mg/kg. The attenuating effects of GPD-1116 and roflumilast seemed to be similar extent at 2 mg/kg; however the effect of GPD-1116 was more potent than these of roflumilast at 0.5 and 1 mg/kg.

Inhibitory Effects of GPD-1116 on CSE+LPS-Induced Lung Histopathological Changes in Guinea Pigs

Fig. 2. Effects of GPD-1116 and Roflumilast on LPS-Induced Acute Lung Injury in Rats

(A) BALF was collected 6 h after LPS inhalation, and the number of neutrophils was counted. Data represent the mean±standard error (S.E.) of 4 rats in each group. (B) BALF supernatants were collected 8 h after LPS inhalation, and an aliquot was mixed to average the gelatinolytic activities in each group. The samples (1.8 µg protein/lane) were analyzed by gelatin zymography. The main band was detected as proMMP-9 with a molecular size of 92 kDa (upper panel). Densitometric analysis of proMMP-9 was performed using Gel-Pro Analyzer ver. 3.1 (lower panel). Typical image of gelatin zymography was shown and the figure below the image represents the mean and S.E. of 3 separate experiments. M: Molecular weight marker, 1: Negative control, 2: LPS control, 3: LPS+GPD-1116 0.5 mg/kg, 4: LPS+GPD-1116 1 mg/kg, 5: LPS+GPD-1116 2 mg/kg, 6: LPS+Roflumilast 0.5 mg/kg, 7: LPS+Roflumilast 1 mg/kg, 8: LPS+Roflumilast 2 mg/kg, MMP: MMP marker (human fibrosarcoma cell supernatant). **: $p<0.01$, Welch’s t-test (versus the negative control group). †: $p<0.05$, ††: $p<0.01$, Dunnet’s multiple comparison test (versus the LPS control group). ‡: $p<0.05$, Steel’s test (versus the LPS control group).
shown in Table 2, some histopathological findings in lung were observed, such as neutrophils and macrophages infiltrated into the alveolus and/or bronchiole, accompanied by thickening of the alveolar wall and the presence of foreign body giant cells, in response to intratracheal instillation of vehicle in the negative group; however, the degree of histopathological change was slight (mean histopathological score: 5.0). In the CSE + LPS control group, similar histopathological changes were observed, but the extent was greater than that observed in the negative control group (mean histopathological score: 13.1). On the other hand, GPD-1116 suppressed these histopathological changes to a certain extent, significantly reducing the mean score to 10.0.

Furthermore, we measured MLI as an index of the histopathological changes induced by CSE plus LPS. Increase in MLI indicates enlargement of alveolus, representing the destruction of alveolus in COPD, which is one of the characteristic findings observed in COPD patients. As shown in Fig. 3, enlargement of the alveolus occurred in response to CSE and LPS, with a significant increase in MLI from 41.6 µm in the negative control group to 61.6 µm in the CSE + LPS control group. GPD-1116 significantly inhibited the enlargement of the alveolus by 54.0%, and it was suggested that GPD-1116 could inhibit the disease progression in this model.

Table 2. Inhibitory Effect of GPD-1116 on Histopathological Findings in CSE + LPS-Induced COPD Model in Guinea Pigs

<table>
<thead>
<tr>
<th>Histopathological findings</th>
<th>Group</th>
<th>Negative control</th>
<th>CSE + LPS control</th>
<th>CSE + LPS + GPD-1116 (1 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Grade</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Trachea Significant lesion</td>
<td></td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bronchus Significant lesion</td>
<td></td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lung Anterior lobe Neutrophil infiltration, alveolus and/or bronchiole</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Macrophage infiltration, alveolus</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Foreign body giant cell, alveolus</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thickening of alveolar wall</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Foreign body, alveolus and/or bronchiole</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Posterior lobe Neutrophil infiltration, alveolus and/or bronchiole</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Macrophage infiltration, alveolus</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Foreign body giant cell, alveolus</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thickening of alveolar wall</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Foreign body, alveolus and/or bronchiole</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

[Mean score] [5.0] [13.7]* [10.0]**

Numerals represent the number of animals with the indicated findings. Grade 0: No lesion, 1: Slight, 2: Moderate, 4: Severe. *: p<0.05, Wilcoxon's rank sum test (versus the negative control group). **: p<0.01, Wilcoxon's rank sum test (versus the CSE + LPS control group).

Inhibitory Effects of GPD-1116 on Antigen-Induced Bi-Phasic Asthma and Lung Eosinophilia in Guinea Pigs

In the OVA control group, sRaw immediately increased after antigen challenge, reaching 498.07±24.12% of the basal value at 1 min after the antigen challenge, and the increase in sRaw disappeared until 2 h after the antigen challenge (Fig. 4A). Following the IAR phase, sRaw gradually increased again, reaching 95.71±12.61% at 6 h after the antigen challenge (Fig. 4B), and the increase in the AUC calculated by sRaw between 4 and 8 h reached by 13.2-fold in comparison with the negative control group (Fig. 4D). In the IAR phase,
GPD-1116 slightly inhibited the increase in sRaw at 0.3 and 3 mg/kg; however, these effects were not statistically significant (Figs. 4A, C). The inhibition rates at doses of 0.03, 0.3, and 3 mg/kg were 1.5, 17.5, and 17.0%, respectively. On the other hand, GPD-1116 significantly reduced the increase in the AUC at doses of 0.3 and 3 mg/kg, and the inhibition rates at 0.03, 0.3, and 3 mg/kg of GPD-1116 were 20.7, 54.6, and 51.3%, respectively (Figs. 4B, D). Montelukast at 3 mg/kg also inhibited the increase in sRaw during both the IAR and LAR phases, with inhibition rates of 31.7 and 61.7%, respectively (Figs. 4A–D).

In this allergic model, the infiltration of a number of eosinophils was counted. Data represent the mean±S.E. of 7 guinea pigs in each group. **: p<0.01, Welch's t-test (versus the negative control group). †: p<0.05, ††: p<0.01, Dunnet's multiple comparison test (versus the OVA control group). ##: p<0.01, Student's t-test (versus the OVA control group). ‡: p<0.05, Steel's test (versus the OVA control group).

Table 3. Inhibitory Effects of GPD-1116 on Increases in Right Ventricular Weight and RVSP Induced by MCT in Rats

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
<th>RV/LV+S</th>
<th>Inhibition (%)</th>
<th>RVSP (mmHg)</th>
<th>Inhibition (%)</th>
<th>SAP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>8</td>
<td>0.30±0.01</td>
<td>—</td>
<td>25.0±3.0</td>
<td>—</td>
<td>149.1±5.2</td>
</tr>
<tr>
<td>MCT control</td>
<td>8</td>
<td>0.53±0.05**</td>
<td>—</td>
<td>63.5±10.5**</td>
<td>—</td>
<td>151.8±6.2</td>
</tr>
<tr>
<td>MCT+GPD-1116 (2 mg/kg)</td>
<td>7</td>
<td>0.42±0.04</td>
<td>47.8</td>
<td>44.3±6.5</td>
<td>49.9</td>
<td>150.3±7.8</td>
</tr>
</tbody>
</table>

Data represent the mean±S.E. **: p<0.01, Welch’s t-test (versus the negative control group).

Table 4. Inhibitory Effects of GPD-1116, Roflumilast and Tadalafil on Increases in Right Ventricular Weight Induced by MCT in Rats

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>RV/LV+S</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>—</td>
<td>5</td>
<td>0.28±0.01</td>
<td>—</td>
</tr>
<tr>
<td>MCT control</td>
<td>—</td>
<td>10</td>
<td>0.50±0.04**</td>
<td>—</td>
</tr>
<tr>
<td>MCT+GPD-1116</td>
<td>2</td>
<td>10</td>
<td>0.38±0.02*</td>
<td>54.5</td>
</tr>
<tr>
<td>MCT+Roflumilast</td>
<td>2</td>
<td>9</td>
<td>0.44±0.03</td>
<td>27.3</td>
</tr>
<tr>
<td>MCT+Tadalafil</td>
<td>10</td>
<td>10</td>
<td>0.43±0.03</td>
<td>31.8</td>
</tr>
</tbody>
</table>

Data represent the mean±S.E. **: p<0.01, Welch’s t-test (versus the negative control group). *: p<0.05, Welch’s t-test (versus the MCT control group).
sinophils into the airway was induced by OVA; significant increase in eosinophils in the BALF was observed compared with that in the negative control group (Fig. 4E). GPD-1116 potently inhibited the eosinophil infiltration, with inhibition rates of 72.0, 63.3, and 77.9% at doses of 0.01, 0.1, and 1 mg/kg, respectively.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>cAMP (pmol/mg protein)</th>
<th>Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>5</td>
<td>25.7±3.0</td>
<td>—</td>
</tr>
<tr>
<td>GPD-1116</td>
<td>2</td>
<td>5</td>
<td>40.3±8.5</td>
<td>56.8</td>
</tr>
<tr>
<td>Roflumilast</td>
<td>2</td>
<td>5</td>
<td>36.9±9.7</td>
<td>43.6</td>
</tr>
<tr>
<td>Tadalafil</td>
<td>10</td>
<td>5</td>
<td>27.0±4.2</td>
<td>5.1</td>
</tr>
</tbody>
</table>

Data represent the mean±S.E.

Inhibitory Effects of GPD-1116 on MCT-Induced Pulmonary Hypertension in Rats As shown in Table 3, MCT-induced pulmonary hypertension was confirmed by the increase in RVSP without changes in SAP, and the increase in RVSP was well correlated with increases in ratio of RV/(LV+S), as reported by Izikki et al. and Sawamura et al.\(^{14,15}\)

Fig. 5. Influence of GPD-1116 on Gastric Emptying and Rectal Temperature in Rats

(A) Glass beads were orally loaded 1 h after the indicated treatments, and gastric emptying was determined 40 min after the load. (B) Rectal temperature was measured after the indicated treatments until 180 min. Data represent the mean±S.E. of 5 rats in each group. †: p<0.05, Dunnet's multiple comparison test (versus the control group). **: p<0.01, Student's t-test (versus the control group). *: p<0.05, Welch's t-test (versus the control group).
GPD-1116 suppressed both the increase in RVSP, with an inhibition rate of 49.9%, and the increase in the ratio of RV/LV+S, with an inhibition rate of 47.8%.

Furthermore, we compared the inhibitory effect of GPD-1116 in this model with those of roflumilast and tadalafil. As described above, the increase in RVSP was well correlated with the increase the ratio of RV/LV+S, we evaluated the efficacy of these compounds with the ratio of RV/LV+S. As shown in Table 4, GPD-1116 demonstrated the inhibitory effect on the increase in ratio of RV/LV+S, with the inhibition rate of 54.5%, and the inhibitory effect of GPD-1116 was more potent than those of roflumilast and tadalafil, with the inhibition rate of 27.3 and 31.8%, respectively.

**Influence of GPD-1116 on Pulmonary cAMP Levels in Rats** As shown in Table 5, basal pulmonary cAMP concentrations in rats treated with vehicle was 25.7±3.0 pmol/mg protein. On the other hand, GPD-1116 and roflumilast were 40.3±8.5 and 36.9±9.7 pmol/mg protein, respectively, and GPD-1116 and roflumilast tended to increase the concentrations of cAMP, with the increase rate of 56.8 and 43.6%, respectively. cAMP level in rats treated with tadalafil was 27.0±4.2 pmol/mg protein, and tadalafil did not show any influence on pulmonary cAMP levels.

**Influence of GPD-1116 on Gastric Emptying and Rectal Temperature in Rats** GPD-1116 and roflumilast suppressed gastric emptying in a dose-dependent manner with similar degree, and the effects of both compounds at 1 mg/kg were statistically significant compared with that of the control group (Fig. 5A). Roflumilast, another PDE4 inhibitor used as a positive control, strongly suppressed gastric emptying at 1 mg/kg, and the degree was more potent than GPD-1116 and roflumilast (Fig. 5A).

In rectal temperature, GPD-1116 did not show any affect 3 mg/kg, while roflumilast moderately lowered rectal temperature at the same dose (Fig. 5B). Moreover, roflumilast exponentially lowered the temperature after the administration at 3 mg/kg.

**Emetic Action of GPD-1116 in Dogs** As shown in Table 6, no vomiting episode was observed at 0.5 and 1 mg/kg of GPD-1116, and total three episodes of vomiting, one episode in each dog, were observed at a dose of 3 mg/kg of GPD-1116. In case of roflumilast, no vomiting episode was observed at a dose of 0.5 mg/kg, and total two episodes of vomiting in 2 of 3 dogs were observed at a dose of 1 mg/kg of roflumilast. In case of rolipram, only one vomiting episode in 1 of 3 dogs was observed at a dose of 0.1 mg/kg, however a number of vomiting episodes were observed in all dogs at a dose of 1 mg/kg. From these observation, the maximum tolerated doses (MTD) of GPD-1116, roflumilast and rolipram were 1, 0.5 and <0.1 mg/kg, respectively.

**DISCUSSION**

As shown in Table 1, GPD-1116 and GPD-1133 predominantly inhibited not only PDE4 subtypes but also PDE1 subtypes, and its activity as a PDE1 inhibitor was remarkable, exhibiting more potent inhibitory activity toward PDE1 subtypes than other compounds known as PDE1 inhibitors, such as 8-MM-IBMX and vinpocetine, which have IC50 values ranging from 1 to 100 μM. This selectivity of GPD-1116 and GPD-1133 for PDE1 and PDE4 is thought to be due to its basic chemical structure ([1,8]naphthyridin-4(5H)-one). For example, KF19514, a PDE inhibitor that shows selectivity for both PDE1 and PDE4, also has the [1,8]naphthyridin-4(5H)-one structure. In contrast, another group of PDE4 inhibitors that includes rolipram and roflumilast has a catechol structure and shows strict selectivity for PDE4. Thus, such structural differences could explain the unique pharmacological profile of GPD-1116.

It has been well known that cAMP signaling have important roles on the regulation of inflammation and smooth muscle tension, and the agents increasing intracellular cAMP levels have been applied as therapeutics for inflammatory or circulatory diseases. For instance, β2 receptor agonists, such as formoterol and indacaterol, which directly increase intracellular cAMP levels via activation of adenyl cyclase, have been used for medicinal therapy for asthma or COPD. Furthermore, roflumilast, which indirectly increases intracellular cAMP levels via inhibition of PDE4, has recently emerged as a therapeutic drug for the treatment of COPD. As shown in this report, GPD-1116 demonstrated a potent inhibitory activity for PDE4 subtypes in vitro (Table 1) and a marked increase in pulmonary cAMP levels in vivo (Table 5), thus we have considered that the efficacy of GPD-1116 in our models of pulmonary diseases would be due to anti-inflammation action predominantly via the inhibition of PDE4 because anti-inflammatory effects by PDE1 inhibition have not been well recognized to date.

It has also been reported that cGMP signaling has many physiological and pathophysiological functions, and some agents increasing intracellular cGMP levels, such as nitroprusside and nitroglycerine (nitric oxide (NO) donors), have been used for cardiac infarction. Furthermore, among PDE iso-enzymes increasing intracellular cGMP levels, PDE5 inhibitors, such as tadalafil, have actually been applied in clinic as therapeutic drugs for erectile dysfunction and pulmonary hypertension. On other hand, the roles and functions of PDE1, which also increases cGMP levels, in the pathogenesis or progression of diseases had not been known well because there were few reports referred to contribution of PDE1 in diseases. Recently, however, some studies have revealed several new findings on the expression and functions of PDE1 in physiologic and pathogenic points of view. For instance, it has been reported that the expression of PDE1 is strongly and consistently observed in lung arterial smooth muscle of patients with pulmonary hypertension. Furthermore, Jeon et al. reported that PDE1A has an important role in β-catenin signaling and regulates proliferation of smooth muscle cell. Moreover, it has been reported that PDE1 inhibitors, such as vinpocetine and IC83640, effectively attenuate the proliferation and migration of vascular smooth muscle cells in vitro. These facts suggest that the agents inhibiting PDE1 might be one of potential therapeutic applications for patients with proliferative diseases of artery smooth muscle, such as pulmonary hypertension. In fact, GPD-1116 showed a significant inhibitory effect on our MCT-induced pulmonary hypertension model, and the efficacy was more potent than that of typical PDE5 inhibitor, tadalafil, which has been used in clinic for pulmonary hypertension (Table 4). Again, one of the features of GPD-1116 is to inhibit not only PDE4 but also PDE1, it would be reasonable to apply GPD-1116 as a therapeutic drug for patients with inflammatory diseases associated with proliferation of artery smooth...
It has been known that the inhibition of PDE4 affects the movement of the gastrointestinal tract, the secretion of gastric acid or the function of the central nervous system \(^{39-41}\), therefore PDE4 inhibitors frequently raise some adverse events, such as nausea, emesis or abdominal discomfort in humans.\(^ {1,2}\)

The efficacy of GPD-1116 in several disease models, with effective doses ranging from 0.3 to 2 mg/kg, was comparable to or more potent than that of roflumilast although the inhibitory activity of GPD-1116 for PDE4 was about 100-fold less potent than that of roflumilast. On the other hand, the influence of GPD-1116 on side effects, such as suppression of gastric emptying and decrease in body temperature, was similar to or less potent than that of roflumilast within the range of same effective doses (Figs. 5A, B), furthermore, MTD of GPD-1116 on emetic action in dogs was higher than that of roflumilast (Table 6) although the effective doses of GPD-1116 and roflumilast in dogs were unknown. These results suggest a possibility that GPD-1116 could be a superior therapeutic drug without side effects. At present, it is unclear why GPD-1116 has the efficacy comparable to roflumilast with less potency on side effects in vivo; however we have presumed that differences in in vivo properties between these compounds, such as pharmacokinetics including absorption and distribution after administration, or membrane permeability in central nervous system and gastrointestinal tract, could contribute to the fewer side effects of GPD-1116 in vivo. Furthermore, we could not deny a possibility that the good balance of GPD-1116 between PDE1 and PDE4 inhibition might be related to our results.

In summary, our results showed that GPD-1116 potently inhibited both PDE4 and PDE1 and had an excellent pharmacological profile in vivo, which might be attributed in part to PDE1 inhibition. Therefore, further studies are required to determine the specific mechanisms of PDE1 inhibition by GPD-1116.

Acknowledgments We thank Dr. Tomoji Aotsuka, Mr. Kentaro Kumazawa, and Ms. Hisae Takayama for synthesizing test compounds. We also thank Dr. Jun-ichi Fuchikami (Fuji Biomedix), Mr. Souta Ushijima (Panapharm Laboratories), Ms. Moe Endo, Ms. Kiri Inoue, and Mr. Yuta Ito for technical assistance.

Conflict of Interest The authors are employees of ASKA Pharmaceutical Co., Ltd.


