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Biochemistry, pharmacokinetics, and toxicology of a potent and selective DPP8/9 inhibitor

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ABSTRACT

DPP-IV (EC 3.4.14.5) is a validated drug target for human type II diabetes. DPP-IV inhibitors without DPP8/9 inhibitory activity have been sought because a possible association has been reported between a "DPP8/9 inhibitor" and severe toxicity in animals. However, at present, it is not known whether the observed toxicity is associated with DPP8/9 inhibition, or an off-target effect induced by the compound. We investigated whether the inhibition of DPP8/9 is the cause of the severe toxicity in animals using a very potent and selective DPP8/9 inhibitor with different pharmacophore, 1G244. By Ki measurement, 1G244 is 15- and 8-fold more potent against DPP8 and DPP9, respectively, than the "DPP8/9 inhibitor". Strikingly, the "DPP8/9 inhibitor" does not penetrate the plasma membrane but remains outside the cells, whereas 1G244 readily enters the cells, even at low doses. By repeatedly exposing Sprague–Dawley rats to 1G244 by intravenous injection for a period of 14 days, we observed no significant toxicological symptoms associated with 1G244. Blood and serum chemistry parameters were all within the normal ranges for the treated animals. Because of the high potency, good membrane penetration and adequate tissue distribution of 1G244, the mild symptoms observed are probably associated with DPP8/9 inhibition.

1. Introduction

Dipeptidyl peptidase 8 (DPP8) and DPP9 belong to the prolyl peptidase family, which also includes DPP-IV (EC 3.4.14.5), fibroblast activation protein (FAP), prolyl oligopeptidase, and others [1]. Among these, DPP-IV is a validated drug target for human type II diabetes [2,3]. Chemical inhibitors of DPP-IV are effective in the treatment of type II diabetes, lowering blood sugar levels and improving beta cell function [4,5]. This inhibition is well tolerated and does not cause hypoglycemia or increase body weight in human [5,6]. Two inhibitors of DPP-IV, sitagliptin and vildagliptin, have been on the market since 2007. Two others, saxagliptin and alogliptin, have been submitted to the US Food and Drug Administration (FDA) as new drug applications.

Compared with well-studied DPP-IV, the functions of DPP8 and DPP9 are unclear. Knockout mice for DPP8 or DPP9 are not available. DPP8 and DPP9 are highly homologous proteins, with 62% sequence identity [7], and both are ubiquitously expressed [8,9]. They are dimeric soluble proteins that localize in the cytosol, with similar amino-dipeptidase activities [10,11]. Recently, it was demonstrated that the expression levels of DPP8 and DPP9 are upregulated in rats with experimentally induced asthma [12]. Despite tremendous efforts in the field, no potent and selective inhibitors of either DPP8 or DPP9 alone are available [13–16]. So far, the most potent and selective inhibitors of both DPP8 and DPP9 reported are 1G244 (compound 1), which we discovered previously, and the DPP8/9 inhibitor (compound 2) (Fig. 1) [13,17].

The potential functions of DPP8 and DPP9 were deduced in an inhibitor study with compound 2. The administration of compound 2 induces severe toxicity and various pathological symptoms, including alopecia, thrombocytopenia, anemia, enlarged spleen and death in rats, and bloody diarrhea in dogs [17]. Based on that study, great emphasis has been given in the search for DPP-IV

Abbreviations: DPP, dipeptidyl peptidase; DPP-IV, dipeptidyl peptidase IV; FAP, fibroblast activation protein; FDA, the US Food and Drug Administration; IV, intravenous; SD, Sprague–Dawley; GLP, Good Laboratory Practice; CC_{50}, cytotoxic concentration; AUC, the area under the curve.

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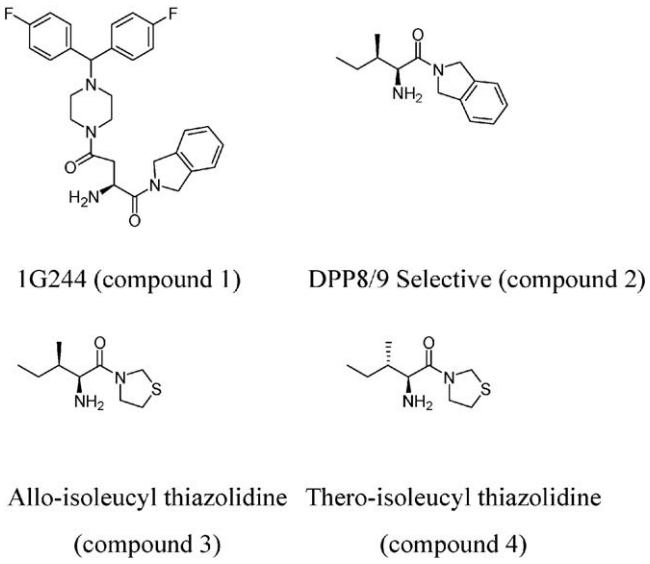

Fig. 1. Chemical structures of DPP8/9 inhibitors.

inhibitors to the "cleanness" of the compound, devoid of any DPP8/9 inhibitory activity. However, at present, it is not known whether the observed toxicity is associated with DPP8/9 inhibition, or whether it is an off-target effect induced by the compound. From the literature, sitagliptin and alogliptin have no DPP8/9 inhibitory activity, whereas vildagliptin has quite potent activities against both DPP8 and DPP9, especially DPP9 [17–19]. Vildagliptin has not yet been approved by the US FDA, though it was approved and on the market in Europe.

It is important to understand whether the inhibition of DPP8/9 is the cause of the severe toxicity observed in animals. A toxicological study with vildagliptin was carried out with high oral doses in a 13-week study to try to answer this question [20]. Vildagliptin is a potent DPP-IV inhibitor and a fairly weak DPP8/9 inhibitor, with Ki values of 810 and 95 nM, respectively [20]. Despite high concentrations of vildagliptin in the blood (above 2 μM), no toxicological consequences were observed with vildagliptin [20]. Because vildagliptin is a highly hydrophilic compound, questions have been raised about how well it penetrates the cell membrane to inhibit cytosolic DPP8/9 [21]. Therefore, whether DPP8/9 inhibition is the direct cause of toxicity in mammals remains to be resolved.

Here we report the characterization of the biochemical and pharmacokinetic properties of 1G244, and the investigation of its toxicological effects through repeated exposure of Sprague–Dawley (SD) rats to 1G244 by intravenous (IV) injection for a period of 14 days. The rats were evaluated with respect to mortality/moribundity, body weight, clinical signs, clinical pathology (hematology and serum chemistry), organ weights and gross necropsy.

2. Materials and methods

2.1. Materials

Strep-Tactin® resin was purchased from EMD Chemicals Inc. (Darmstadt, Germany). The enzyme substrates Ala–Pro–pNA and Gly–Pro–pNA were purchased from Bachem (Torrance, CA, USA). 1G244 was synthesized by Ryss Inc. (Taipei, Taiwan), as described previously [13]. Compound 2 was synthesized according to Lankas et al. [17]. Principles of laboratory animal care' (NIH publication no. 85–23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm) were followed.

2.2. Expression and purification of human recombinant DPP-IV, DPP8, DPP9, DPP2 and FAP proteins

DPP8, DPP9, DPP-IV and FAP were expressed and purified as described previously [11,22–26]. Recombinant DPP2 protein was purified as described previously, with some modifications [27,28]. The purity of the protein was confirmed by SDS-PAGE with Coomassie blue staining. The protein concentrations were determined with the Bradford method using bovine serum albumin as the standard.

2.3. IC_{50} determination

The assay was carried out essentially as described previously [13,29–32]. More specifically, enzyme activities were assayed in a total volume of 100 μL for 30 min at 37 °C at an emission wavelength of 405 nM with a Power Wave X spectrometer (Bio-Tek Instrument, Inc., Winooski, VT, USA). The IC_{50} values for DPP8, DPP9 and FAP were determined in phosphate-buffered saline (PBS, pH 8.0), in the presence of 2.5 mM Gly–Pro–pNA, 1.5 mM Gly–Pro–pNA and 1.5 mM Ala–Pro–pNA, respectively. The IC_{50} for DPP-IV was determined in 2 mM Tris–HCl (pH 8.0) in the presence of 500 μM Gly–Pro–pNA. The IC_{50} for DPP2 was determined in PBS (pH 5.5), in the presence of 1.5 mM Gly–Pro–pNA. The inhibitor concentrations ranged from 100 to 0.003 μM. IC_{50} values were computed with commercially available curve-fitting programs such as SigmaPlot.

2.4. Inhibition constant (Ki) measurement

The inhibitory activity of 1G244 against DPP8 or DPP9 was determined from its ability to inhibit the hydrolysis of Ala–Pro–pNA. Ki was measured as described previously, with a Beckman DU 800 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA) [28,33]. The assay was performed in PBS in a total volume of 100 μL with 10 nM DPP8 or 10 nM DPP9. For the slow-tight binding assay of DPP8 with 1G244, the concentration of the substrate was 125 μM, and the concentrations of 1G244 ranged from 0 to 12.5 μM. For the competitive binding assays, the substrate and inhibitor concentrations were 62.5–500 μM and 0–50 μM for DPP8 with compound 2; 93.75–750 μM and 0–30 nM for DPP9 with 1G244; and 93.75–750 μM and 0–200 nM for DPP9 with compound 2, respectively. The hydrolysis of the Ala–Pro–pNA substrate was monitored continuously by measuring the light emission at 405 nM for 3–5 min. The light intensity was corrected using a standard curve. The data were fitted to slow-binding inhibition and competitive inhibition as described previously [28,33].

2.5. Mammalian cell culture and inhibitor uptake

The mammalian cells were cultured with a standard protocol suggested by the American Type Culture Collection (ATCC). To measure the inhibitor uptake into the mammalian cells, human embryonic kidney (HEK) 293T cells were cultured in 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM) or serum-free DMEM (Invitrogen, Carlsbad, CA, USA). The inhibitors were dissolved in dimethyl sulfoxide (DMSO), added to the culture medium, and incubated with the cells for 6 h. The final DMSO concentration was less than 1%. After treatment, the cells were collected and washed five times with PBS. The cells were lysed in lysis buffer containing 142.5 mM KCl, 5 mM $MgCl_2$, 10 mM Hepes (pH 7.4), 1 mM EGTA and 0.2% NP40 on ice for 15 min. The cell lysates were clarified and collected, and the total protein concentrations were quantified with the Bio-Rad Protein Assay Kit (Bio-Rad laboratories, Hercules, CA, USA). Equal amounts of protein

(20 μg) in the clarified cell lysates were loaded onto a 96-well plate and the activities of the enzymes were measured with Ala–Pro–pNA (2 mM) by detecting pNA at OD_{405}. Percentage inhibition was measured by comparison with the activity of the no-inhibitor control (cells treated with an equivalent amount of DMSO only).

2.6. Pharmacokinetic analysis of 1G244 in Sprague–Dawley rats

The SD rats for the pharmacokinetic study were obtained from BioLASCO Taiwan Co., Ltd. (Ilan, Taiwan, ROC), and housed in the animal facility at the National Health Research Institutes, Taiwan, ROC. The animal studies were performed according to committee-approved procedures. Male rats, each weighing 330–380 g (9–10 weeks old), were quarantined for 1 week before use. The animals were surgically implanted with a jugular-vein cannula 1 day before treatment, and were fasted before treatment. 1G244 was given to the rats ($n = 4$) as an intravenous (1 mg/kg) or oral (2 mg/kg) dose prepared in a mixture of DMSO/Cremophor/water (5/10/85, v/v/v). The volume of the dosing solution given was adjusted according to the body weight recorded before the drug was administered. At 0 (immediately before dosing), 2, 5 (intravenous only), 15 and 30 min and 1, 2, 4, 6, 8, 12 and 24 h after dosing, a blood sample (~150 μL) was taken from each animal via the jugular-vein cannula and stored in ice (0–4 °C). The processing of the plasma and analysis by high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) were carried out as described [34]. The plasma concentration data were analyzed with a standard noncompartmental method with the Kinetica software program (InnaPhase, Philadelphia, PA, USA).

2.7. Rat toxicity studies

For the toxicological study, SD rats aged 3–4 weeks were purchased from Harlan Sprague Dawley, Inc., USA. The animals were housed and the experiments were conducted in the animal facility of Level Inc. (Taipei, Taiwan, ROC), a Good Laboratory Practice (GLP)-certified facility. The animals were quarantined for 7 days and acclimated for 21 days in the testing room before treatment. At 7–8 weeks of age, male and female rats weighing 150–250 g were randomly assigned to one of four groups with four males and four females in each group. 1G244 was prepared fresh daily by dissolving it in sterile 75% polyethylene glycol (PEG) 300. The rats were administered with vehicle (sterile 75% PEG300) or 1G244 (1, 10, or 30 mg/kg per day) intravenously at a dose volume of 1 mL/kg through the tail vein. Daily observations were made for physical signs of toxicity during the study period. All animals surviving at the end of the study period and all moribund animals during the study period were anesthetized with ketamine/xylazine, their blood was sampled, and they were killed, after which a complete necropsy was conducted. The visceral organs were dissected from all the rats, weighed, and fixed in 10% buffered formalin. These samples were processed with routine histological methods for pathological examination. Blood samples for hematology and serum chemistry analysis were collected before the study and on the day upon which the animals were killed. The blood samples were analyzed for hematological parameters with an Automated Hematology Analyzer (Model Xt-1800i, Sysmex Co. Ltd., Kobe, Japan) and serum chemistry was analyzed with a Model 7080 Automatic Analyzer (Hitachi, Ltd., Tokyo, Japan).

2.8. Data analysis

The data for all parameters were calculated and expressed as means ± standard deviations or percentages. Data were deemed statistically significant when the P values were lower than 0.05 ($P < 0.05$) on one-way ANOVA.

3. Results

Based on the report by Lankas et al. [17], a possible link was inferred between DPP8/9 inhibition and cellular toxicity. The administration of either compound 2 or allo-isoleucyl thiazolidine (compound 3) to rats and dogs resulted in similar pathological symptoms in two-week toxicity studies [17]. However, threo-isoleucyl thiazolidine (compound 4) does not cause any of these symptoms at similar dose, either because compound 4 is a weaker inhibitor of DPP8/9 (Table 1), or due to other unknown reasons [17].

We used the P1 and P2 sites to describe the different pharmacophores of the compounds, as used for the assignment of the enzyme substrates. We ascertained in our previous study that, in inhibiting DPP8, the isoindoline moiety is preferred over other five-ring structures at the P1 site [13]. Both compounds 2 and 3 have an allo-isoleucyl moiety at the P2 site (Fig. 1). However, compound 4 has a different P2 site (the threo-isoleucyl moiety), raising the possibility that the toxicity is either related to the P2 site or to the chemical structure of the compounds. Because 1G244 (compound 1) has a different P2 site from those of compounds 2, 3, and 4, it provides an opportunity to investigate whether the observed toxicity is related to the chemical compound per se or to the protein target (DPP8/9).

3.1. Biochemical properties of 1G244

1G244 has previously been reported to be a potent and selective DPP8 inhibitor [13]. Here, we characterized the potency and selectivity of both 1G244 and compound 2 against the DPP enzymes in our assay system (Section 2). 1G244 had the IC_{50} values of 14 and 53 nM against DPP8 and DPP9, respectively (Table 1). It did not inhibit DPP-IV, FAP, or DPP2, with IC_{50} values greater than 100 μM. Under the same conditions, compound 2 had IC_{50} values of 145 and 242 nM against DPP8 and DPP9, respectively (Table 1). The Ki values for 1G244 were 0.9 and 4.2 nM for DPP8 and DPP9, respectively (Fig. 2A and B). Interestingly, 1G244 was a slow-tight binding competitive inhibitor of DPP8 (Fig. 2A), but it was a competitive and reversible inhibitor of DPP9 (Fig. 2B). Compound 2

Table 1
Potency and selectivity of DPP8/9 inhibitors.

Cmpd	Name	IC_{50} (nM)					Ki (nM)		Reference
		DPP8	DPP9	DPP-IV	FAP	DPP2	DPP8	DPP9	
1	1G244	14	53	>100,000	>100,000	>100,000	0.9	4.2	Jiaang et al. [13] and this study
2	DPP8/9 selective	145	242	>100,000	>100,000	>100,000	13.7	33.7	This study
		38	55	30,000	>100,000	14,000			Lankas et al. [17]
3	Allo-isoleucyl thiazolidine	220	320	460	>100,000	18,000			Lankas et al. [17]
4	Threo-isoleucyl thiazolidine	2180	1600	420	>100,000	14,000			Lankas et al. [17]

FAP, fibroblast activation protein.

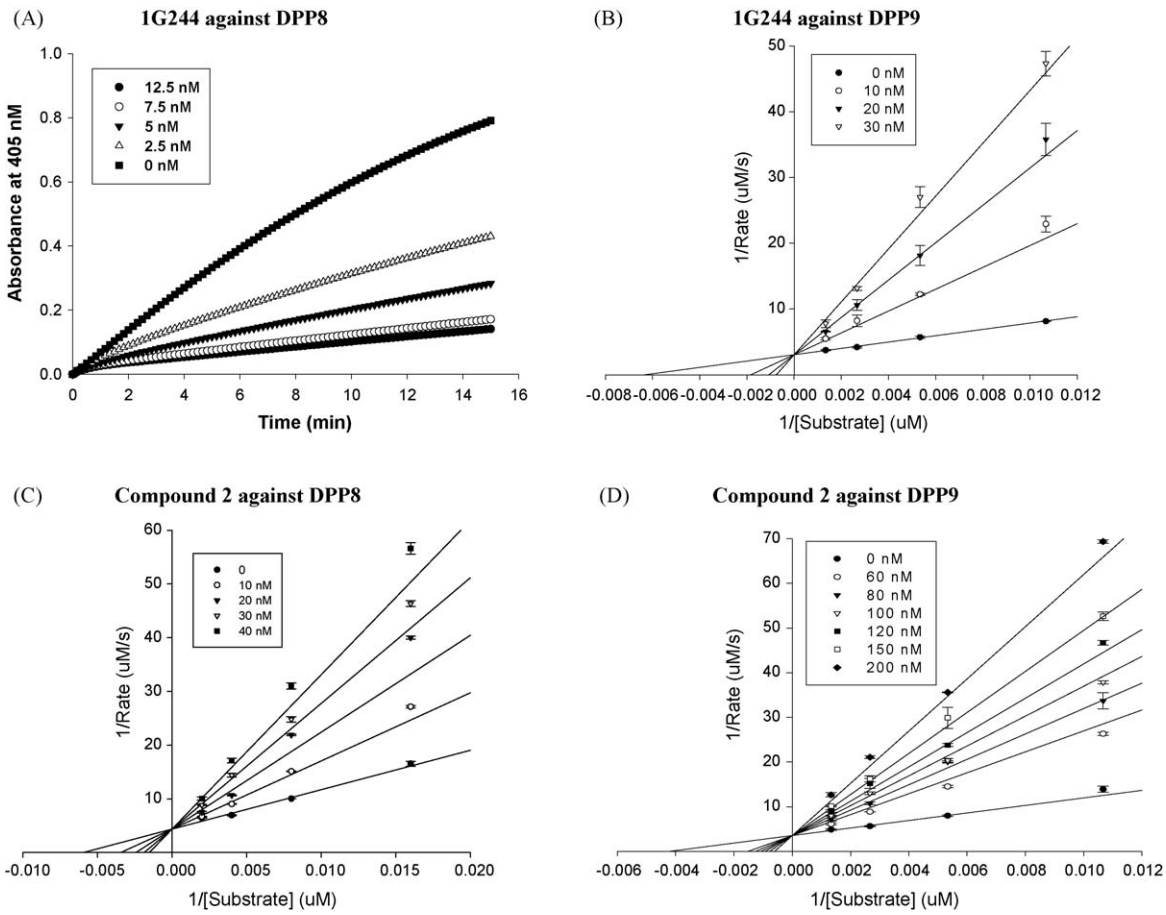

Fig. 2. Determination of the Ki values for 1G244 and compound 2 against DPP8 and DPP9. (A) Slow-binding kinetics for the inhibition of DPP8 by 1G244. (B) Competitive inhibition of DPP9 by 1G244. (C) Competitive inhibition of DPP8 by compound 2. (D) Competitive inhibition of DPP9 by compound 2. The concentrations of the inhibitors represented by different symbols are shown in the inserts.

was a competitive inhibitor of both DPP8 and DPP9, with Ki values of 13.7 and 33.7 nM, respectively (Fig. 2C and D). 1G244 showed minimal cytotoxicity against several mammalian cancer cell lines, with CC_{50} (cytotoxic concentration) values well over 20 μM (Table 1S). Therefore, 1G244 is about 15- and 8-fold more potent than compound 2 in inhibiting DPP8 and DPP9, respectively.

Because DPP8 and DPP9 are cytosolic enzymes, their inhibition can only be achieved if the compound penetrates the plasma membrane and is taken up by the cells. To determine whether the inhibitors pass through the plasma membrane and enter the cells, we incubated cells with the inhibitors for 6 h before collecting and lysing the cells to measure their endogenous DPP activity with the substrate Ala–Pro–pNA. As shown in Fig. 3 (gray bars), if incubated directly with total cell lysate, both 1G244 and compound 2 inhibited the DPP activity to around 40% of the total activity, consistent with the presence of other DPP enzymes inside the cells. Next, we incubated the inhibitors with cells and then collected and lysed the cells. As shown in Fig. 3A and C, the inhibition of DPP8/9 activity by 1G244 was dose dependent. Moreover, the inhibition was detectable at concentrations as low as 320 nM. The complete inhibition of DPP8/9 activity was observed at a 1G244 concentration of only 8 μM outside the cells (Fig. 3A and C). Because high concentration of 1G244 (>20 μM) resulted in cell lysis in the absence of the serum, up to 8 μM was used in the assay (Fig. 3C). Moreover, we have determined the relative amount of 1G244 inside the cells by incubating the cells with 1G244 at 20 μM for 2 h, which was sufficient for the entry (data not shown). Around 91% of the compound was detected by HPLC–MASS quantification (Section 2) proving that 1G244 indeed readily entered inside the cells (data not shown). These results indicate that 1G244 passes through the membrane and enters the cell to inhibit DPP8/9. Strikingly, in comparison, under the same conditions, no inhibition of DPP activity was observed with compound 2, even at 150 or 300 μM (Fig. 3B and D). The extent of the inhibition was similar in the presence or absence of 10% serum, indicating that protein binding was not an issue here (Fig. 3). These results indicate that compound 2 does not penetrate the plasma membrane at all. Thus, the toxicological effects of compound 2 reported by Lankas et al. are most likely associated with an off-target effect, targeting protein(s) outside the cells or on the plasma membrane. In summary, 1G244 readily penetrates the plasma membrane to inhibit DPP8/9 enzymes inside the cell, whereas compound 2 does not. These results are consistent with the lipophilic nature of 1G244 (Fig. 1).

3.2. Pharmacokinetic properties of 1G244

To use this compound for animal studies, we performed a pharmacokinetic study to determine the oral bioavailability and other parameters of 1G244 in rats (Table 2). After the intravenous administration of 1G244 at 1.1 mg/kg, the concentration of the parent drug in the plasma was detectable for up to 24 h after administration. The total body clearance was 23.4 ± 0.6 mL/(min kg). The volume of distribution at steady state (V_{ss}) was 3.2 ± 0.4 L/kg, suggesting a wide distribution of the compound to the extravascular tissues. The apparent elimination half-life ($t_{1/2}$) was long, at 7.0 h. After oral administration, the drug was rapidly absorbed. C_{max} (0.2 ± 0.03 nmol/mL) was reached 1 h after admin-

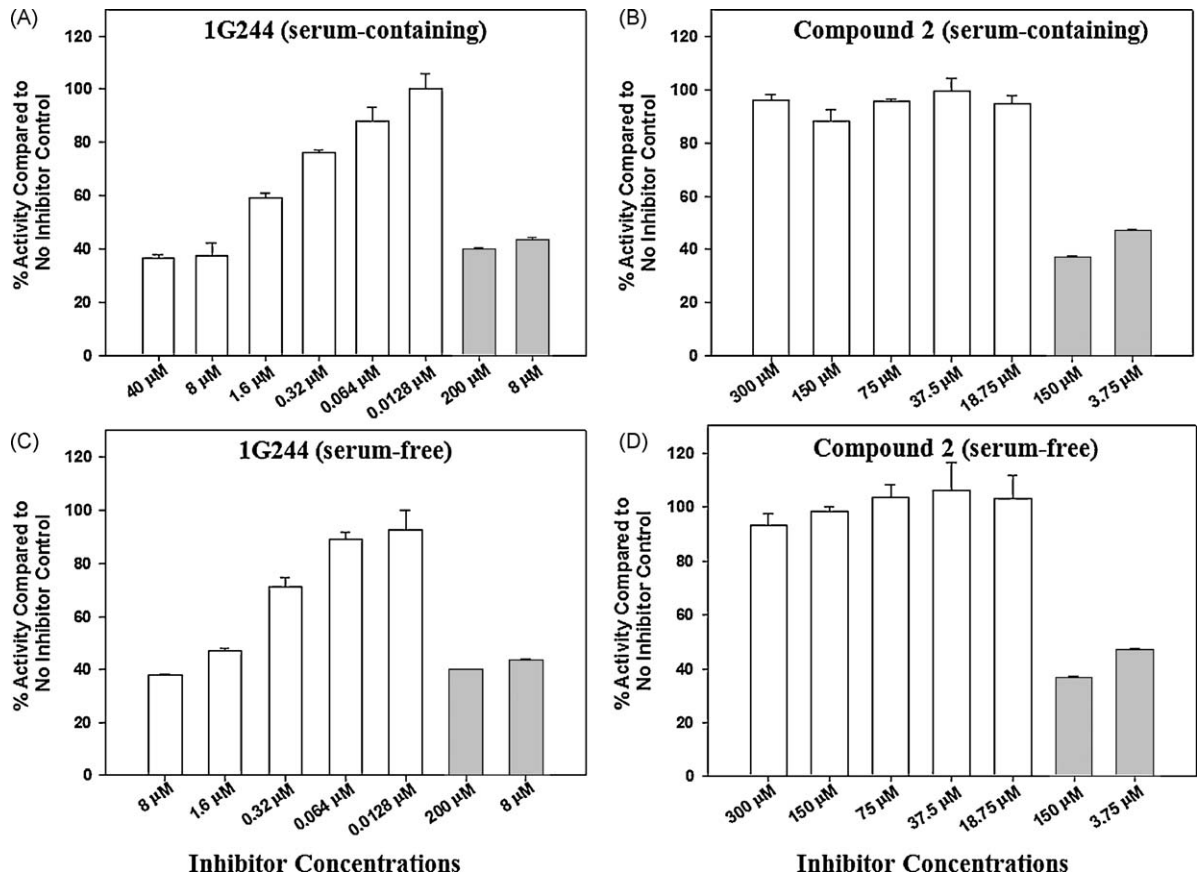

Fig. 3. Uptake of 1G244 and compound 2 by HEK293T cells. The *x*-axis represents the concentration of the compounds used and the *y*-axis shows the activities measured with Ala–Pro–pNA. Gray bars represent the inhibition of DPP activity by the addition of the inhibitor directly to total cell lysates. White bars are the DPP activities after the cells were treated with the compounds for 6 h before the cells were harvested to measure the activities. Panels A and C: treatment with 1G244. Panels B and D: treatment with compound 2. Panels A and B are the DPP activities of 293T cells after treatment with different concentrations of 1G244 and compound 2, respectively, in serum-containing medium. Panels C and D are the same as panels A and B, respectively, except that serum-free medium was used.

istration. The plasma concentration of 1G244 was measurable up to 8 h after administration and averaged 0.003 ± 0.0008 nmol/mL. The oral bioavailability of 1G244 in rats was estimated to be 16.5%.

3.3. Two-week toxicity studies of 1G244 in SD rats

To maximize the tissue exposure to 1G244, we injected the compound intravenously through the tail veins to evaluate its toxicity in SD rats. Doses of 60 or 100 mg/kg resulted in the immediate death of the rats. Lower doses were therefore used for this study, of 1, 10, and 30 mg/kg per day, referred to as the low-, mid-, and high-dose treatments, respectively. Thirty-two Sprague–Dawley rats were randomly assigned to one of four groups, and each group consisted of four males and four females.

1G244 injected intravenously at 30 mg/kg per day caused a startle reflex, clonic and tonic convulsions and opisthonos on the first or second day, whereas only the startle reflex was observed in the first few days in the mid-dose (10 mg/kg per day) group after administration of the drug. No obvious clinical symptoms were observed in the low-dosage group (1 mg/kg per day), except cyanosis starting on day 10. The two-week treatment was completed for the low-dose group. Because of severe cyanosis, the animals of the high-dose group were humanely killed on day 4 (male rats) or day 5 (female rats), and on day 6 for the mid-dose group. Overall, no significant weight loss was observed for any of the animals during the treatment period or on the day of necropsy (data not shown). One female rat in the control group was found dead before the end of the treatment period, and its kidneys were larger than those of the other female rats.

Blood samples were taken from each animal before the experiment started and on the day the animals were killed. Statistical analysis was carried out by comparing the dosed groups with the corresponding vehicle-treated control group. Interestingly, we found that all the hematological and serum parameters for all the treated animals were within the normal animal physiological ranges before and after the experiments. A slight reduction in the red blood cells and hemoglobin of the mid- and high-dose males was observed, compared with those of the normal male controls (Table 3) and of the same animals before treatment (data not shown). This was not observed in the corresponding female animals (Table 3). Moderately higher neutrophil and lower lymphocyte counts were observed among female rats in all dosage groups compared with both the control female group (Table 3) and the same animals before treatment (data not shown), and these

Table 2
Pharmacokinetic properties of 1G244 in rats.

Dose (mg/kg)	Clearance (mL/(min kg))	Vd_{ss} (L/kg)	$t_{1/2}$ (h)	AUC (nmol/(mL h))	C_{max} (nmol/mL)	Oral bioavailability (%)
1.1 (i.v.)	23.4	3.2	7.0	1.6	–	–
2.2 (p.o.)	–	–	1.0	0.5	0.2	16.5

Table 3
Hematological parameters in dosed rats.

Parameter	Vehicle		14-day 1G244 (1 mg/kg)		6-day 1G244 (10 mg/kg)		1G244 (30 mg/kg)	
	M	F[a]	M	F	M	F	M (4-day study)	F (5-day study)
White blood cell ($10^3/\mu L$)	12.2 ± 1.9	7.6 ± 5.4	11.3 ± 0.4	9.5 ± 3.9	13.9 ± 4.0	10.3 ± 4.8	11.8 ± 2.4	9.3 ± 3.5
Red blood cell ($10^6/\mu L$)	7.4 ± 0.5	6.1 ± 0.8	7.2 ± 0.2	6.6 ± 0.3	6.5 ± 0.2*	6.1 ± 0.3	5.9 ± 0.3**	6.1 ± 0.8
Hemoglobin (g/dL)	14.3 ± 0.9	11.9 ± 1.2	13.7 ± 0.5	12.6 ± 0.6	13.0 ± 0.2*	12.1 ± 0.6	11.6 ± 0.4**	11.9 ± 1.4
Hematocrit (%)	42.6 ± 2.3	37.0 ± 1.6	41.5 ± 1.4	38.0 ± 1.6	39.7 ± 1.1	37.1 ± 1.3	35.9 ± 1.1	36.3 ± 3.4
Mean corpuscular volume (fL)	57.3 ± 2.0	61.5 ± 5.5	57.8 ± 1.0	57.9 ± 0.6	61.2 ± 0.5**	61.0 ± 1.2	61.1 ± 2.1*	59.2 ± 2.1
Mean corpuscular hemoglobin (pg)	19.2 ± 0.5	19.7 ± 0.7	19.1 ± 0.3	19.2 ± 0.3	20.1 ± 0.2*	19.8 ± 0.6	19.8 ± 0.5	19.4 ± 0.6
Mean corpuscular hemoglobin concentration (g/dL)	33.5 ± 0.5	32.2 ± 1.7	33.0 ± 0.4	33.1 ± 0.4	32.8 ± 0.3	32.5 ± 0.7	32.4 ± 0.5*	32.8 ± 0.7
Platelet ($10^3/\mu L$)	1124 ± 168	1311 ± 398	1189 ± 199	1170 ± 229	1358 ± 276	1404 ± 62	1023 ± 62	1293 ± 179
Neutrophil (%)	26.0 ± 11.2	11.3 ± 2.6	27.5 ± 4.6	30.9 ± 4.5**	18.1 ± 5.8	34.0 ± 8.7**	20.5 ± 8.8	30.0 ± 7.1**
Lymphocyte (%)	70.4 ± 12.1	86.9 ± 2.8	68.6 ± 5.9	65.6 ± 5.9**	77.7 ± 6.5	63.9 ± 7.7**	76.3 ± 10.5	66.6 ± 7.6**
Monocyte (%)	3.2 ± 0.8	1.7 ± 0.4	3.7 ± 1.3	3.2 ± 1.4	4.0 ± 0.9	1.9 ± 1.3	3.1 ± 2.3	3.3 ± 1.9
Eosinophil (%)	0.4 ± 0.4	0.1 ± 0.2	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1
Basophil (%)	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.2	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0

M: male; F: female.
[a] $n = 3$ (one animal was found dead, no blood sample collected).
* $p < 0.05$.
** $p < 0.01$.

difference were not observed in the corresponding males (Table 3). Again, these values were still within the normal physiological ranges of the animals.

The serum chemistry analysis showed mild reductions in albumin and urea in the female rats, compared with either the control female group (Table 4) or the same group of animals before treatment (data not shown). However, the differences were not outside the normal physiological ranges and did not have a clear association with either the sex of the animal or the dose given (Table 4). Pathological examinations revealed no obvious differences in organ morphologies, including those of the kidneys, spleen, liver, heart and lungs (data not shown). There was an increase in the liver wet weight of the male rats in both the mid- and high-dose groups (Table 5). Slight reductions in the weight of the hearts in the female low-dose group and of the lungs in the male high-dose group were also observed (Table 5). None of these changes were clearly associated with the drug treatments.

4. Discussion

In this study, we have characterized the biochemical properties of an extremely potent and selective DPP8/9 inhibitor, 1G244, and evaluated its toxicological consequences. At all doses of 1G244, we did not observe the reported pathological symptoms associated with compound 2, such as alopecia, thrombocytopenia, reticulocytopenia, or splenomegaly [17]. All the hematological parameters and serum chemistry of all the animals treated with 1G244 were within the normal ranges. Some mild symptoms, including slight reduction in red blood cells, lymphocytes or hemoglobin, or slight increase of neutrophils, seemed to be associated with either male or female animals at certain dosage, but not both. Again, the differences were not outside the normal physiological ranges and did not have a clear association with either the sex of the animal or the dose given. In addition, we observed no organ or tissue abnormalities associated with 1G244.

Table 4
Serum chemistry in dosed rats.

Parameter	Vehicle		14-day 1G244 (1 mg/kg)		6-day 1G244 (10 mg/kg)		1G244 (30 mg/kg)	
	M	F[a]	M	F	M	F	M (4-day study)	F (5-day study)
Aspartate aminotransferase (U/L)	185.7 ± 87.7	115.2 ± 42.4	120.9 ± 17	121.1 ± 31	122.1 ± 30.9	114.7 ± 9.8	87.5 ± 14.7	120 ± 33.8
Alanine aminotransferase (U/L)	84.9 ± 5.8	62.7 ± 14.1	77.9 ± 9.6	56.1 ± 14.7	68.9 ± 18.2	60.5 ± 11.4	55.2 ± 6.2**	65.2 ± 9.4
Glucose (mg/dL)	197 ± 11	204.9 ± 43.5	215.2 ± 40.7	185.8 ± 25.6	246.9 ± 42.3	154.3 ± 17.3	209.5 ± 25.4	178.1 ± 11.8
Total protein (g/dL)	5.5 ± 0.2	5.6 ± 0.4	5.2 ± 0.2	5.1 ± 0.2	5.5 ± 0.1	5.4 ± 0.1	5.5 ± 0.1	5.1 ± 0.7
Albumin (g/dL)	3.3 ± 0.4	3.5 ± 0.4	3 ± 0.3	2.7 ± 0.3*	2.9 ± 0.1	2.8 ± 0.2*	2.8 ± 0.1*	2.7 ± 0.4*
Total bilirubin (mg/dL)	0.02 ± 0.01[a]	0.01 ± NA[b]	0.01 ± 0.0*	NA	0.01 ± 0.00[c]	0.01 ± NA[b]	0.02 ± NA	NA
Urea (mg/dL)	24.9 ± 3	25.8 ± 3.7	26.1 ± 4.1	23.2 ± 3	23.9 ± 1.3	20.2 ± 1.2*	24.4 ± 1.8	18 ± 1**
Creatinine (mg/dL)	0.4 ± 0	0.6 ± 0	0.5 ± 0.1	0.5 ± 0***	0.5 ± 0*	0.6 ± 0.1	0.5 ± 0*	0.5 ± 0
Gamma-glutamyl transferase (U/L)	1 ± 0.2[a]	0.5 ± 0.4	0.4 ± 0.2*	0.7 ± 0.3[a]	0.5 ± 0.4	1.1 ± 0.9	0.8 ± 0.6	0.8 ± 0.5
Alkaline phosphatase (U/L)	522.7 ± 183.1	564.4 ± 129.6	547.6 ± 158.2	633.5 ± 273.8	550.2 ± 64.2	493.2 ± 45.8	551.4 ± 85.2	614.3 ± 96.5
Cholesterol (mg/dL)	90.7 ± 14.5	98.7 ± 1.2	90.6 ± 8.5	99.2 ± 11.5	86.1 ± 8.2	101.7 ± 13.4	103.8 ± 21.5	98.3 ± 18.7
Triglyceride (mg/dL)	21.8 ± 7	29.6 ± 12.4	17.3 ± 3.3	28.9 ± 3.7	56.7 ± 18.4*	36.8 ± 10.1	36.7 ± 10.2	37.9 ± 10.7
Calcium (mg/dL)	9.7 ± 0.3	10.3 ± 0.7	9.6 ± 0.2	10.1 ± 0.4	9.5 ± 0.2	9.9 ± 0.1	10.1 ± 0.3	9.9 ± 0.6
Phosphorus (mg/dL)	8.2 ± 0.6	7.3 ± 1.1	7.9 ± 0.3	7.4 ± 1.8	7.9 ± 0.3	7.9 ± 0.7	7.9 ± 1.2	7.2 ± 0.9
Creatine kinase (U/L)	1007.6 ± 519.6	901.6 ± 646.6	629.0 ± 86.4	766.4 ± 120.6	975.5 ± 332	771.2 ± 171.6	722.1 ± 247.9	433.9 ± 150.3
Amylase (U/L)	1453 ± 403	1083 ± 82	1419 ± 196	944 ± 125	1447 ± 225	1067 ± 227	1189 ± 141	1036 ± 48
Sodium (mmol/L)	141.9 ± 0.8	143.1 ± 1.2	142.9 ± 1.9	141.8 ± 0.6	142 ± 1.2	142.8 ± 0.4	143.3 ± 1	142.8 ± 2.1
Potassium (mmol/L)	4.92 ± 0.3	5.16 ± 1.03	4.58 ± 0.07	4.94 ± 0.8	4.8 ± 0.3	4.7 ± 0.2	4.8 ± 0.3	4.37 ± 0.2
Chloride (mmol/L)	102 ± 2.5	106.1 ± 0.3	104.4 ± 1.5	104.2 ± 1.4	103.2 ± 0.9*	103.9 ± 1	105.7 ± 1.9	105 ± 1.4

M: male, F: female. NA, not available due to detection limit or insufficient number as indicated by b ($n = 1$) or c ($n = 2$).
[a] $n = 3$.
[b] $n = 1$.
[c] $n = 2$.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 5
Organ weights in dosed rats.

Parameter	Vehicle		14-day 1G244 (1 mg/kg)		1G244 (10 mg/kg)		1G244 (30 mg/kg)	
	M	F[a]	M	F	M	F	M	F
Kidneys	2.01 ± 0.05	1.6 ± 0.2[a]	2.06 ± 0.2	1.4 ± 0.07	2.0 ± 0.1	1.5 ± 0.2	2.02 ± 0.1	1.3 ± 0.2
Spleen	0.8 ± 0.1	0.7 ± 0.3[a]	0.8 ± 0.04	0.7 ± 0.05	0.8 ± 0.06	0.7 ± 0.1	0.8 ± 0.07	0.6 ± 0.1
Liver	9.08 ± 0.8	7.3 ± 0.5[a]	8.8 ± 0.2	6.6 ± 0.3	10.08 ± 0.3*	6.9 ± 0.6	10.8 ± 0.8*	6.4 ± 0.7
Heart	1 ± 0.07	0.8 ± 0.04[a]	1.03 ± 0.1	0.7 ± 0.02**	1.0 ± 0.07	0.8 ± 0.09	1.0 ± 0.07	0.7 ± 0.1
Lung	1.8 ± 0.2	1.4 ± 0.2[a]	1.8 ± 0.3	1.3 ± 0.2	1.6 ± 0.04	0.7 ± 0.1	1.4 ± 0.1*	1.3 ± 0.08

M, male; F, female.
[a] $n = 3$.
* $p < 0.05$.
** $p < 0.01$.

Importantly and unexpectedly, we found that compound 2 does not penetrate the plasma membrane of mammalian cells at very high concentrations (Fig. 3C and D). At concentrations as high as 150 or 300 μM in serum-containing or serum-free medium, little DPP inhibition inside the cells was observed (Fig. 3C and D). This indicates that compound 2 remains largely outside the cells, despite its high volume of distribution in the extravascular tissues [17]. Therefore, the toxicological effects observed with compound 2 are not the result of DPP8/9 inhibition. Instead, they most likely occur when compound 2 reacts with a secreted or membrane-associated protein outside the cells, a so-called "off-target" effect through an unknown mechanism.

1G244 is a much more potent DPP8/9 inhibitor than compound 2, with an excellent membrane penetration capacity. No significant toxicological symptoms are associated with DPP8/9 inhibition. 1G244 is 15- and 8-fold more potent than compound 2 against DPP8 and DPP9, respectively (Table 1). Importantly, unlike compound 2, 1G244 readily penetrates the mammalian cell membrane, detectable at 1G244 concentrations as low as 320 nM (Fig. 3A and C). Moreover, maximum inhibition is easily achieved with 8 μM 1G244 (Fig. 3). This concentration is much lower than 28 μM, the in vivo concentration of the drug administered at 1 mg/kg (if the volume of the blood is taken to be 7 mL per 100 g body weight). Therefore, the complete inhibition of DPP8/9 activities should be achieved with 1 mg/kg dose in vivo. The toxicological symptoms caused by 1G244 and compound 2 are consistently different, explainable by the different cellular locations of the inhibitors and their protein targets.

The toxicological symptoms reported with compound 2 were apparent at 10 mg/kg (splenomegaly) and 30 mg/kg (thrombocytopenia and reticulocytopenia) [17]. At 10 and 30 mg/kg, the areas under the curve (AUCs) for 1G244 were 15.6 and 46.9 nmol/(mL h), respectively (Table 2), roughly half the AUC with compound 2 at the same doses (26.4 and 91.1 nmol/(mL h), respectively) [17]. Therefore, the drug exposure is adequate for 1G244. We also performed a pilot study with oral doses of 100 mg/kg ($n = 2$) and 200 mg/kg ($n = 2$) of 1G244 for 14 days in Sprague–Dawley rats. No pathological symptoms were observed in the treated animals or their organs (data not shown). The hematological and serum parameters were all normal like the controls (data not shown). At these concentrations, the in vivo drug concentration is well over 10 μM, which should completely inhibit the DPP8/9 activities, after the AUC and the long half life of 1G244 are taken into account (Table 2). Therefore, both the intravenous and oral dosing experiments consistently demonstrate that the inhibition of DPP8/9 by 1G244 did not result in severe toxicity. One can conclude from these results that DPP8/9 inhibition does not lead to severe toxicological effects as previously reported [17].

Intriguingly, we found that 1G244 is a slow-tight binding inhibitor of DPP8, and a reversible competitive binding inhibitor of DPP9. These different inhibition mechanisms suggest that the binding sites and interaction modes of the enzymes with the inhibitors differ. Therefore, it may be possible to discover a selective DPP8 or DPP9 inhibitor through chemical optimization, based on differences in the inhibitor binding sites. The resolution of the crystal structures of DPP8 and DPP9 in the future will greatly enhance our understanding of the structural and functional relationships of the enzymes and the discovery of inhibitor drugs.

Our study provides important information on the relationship between DPP8/9 inhibition and cellular physiology. At present, little is known for DPP8 and DPP9 regarding their biological functions or their in vivo substrates. No knockout mice study for DPP8 or DPP9 has been reported. Recently, we show that DPP8 and DPP9 are ubiquitously expressed in fibroblasts, epithelials and blood cells [23]. Moreover, the expression levels of DPP8 and DPP9 are not changed before and after phytohaemaglutinin-stimulated Jurkat and peripheral blood mononuclear cells [23], suggesting that they might not play critical roles in the immune function as previously proposed [9,12]. Because 1G244 is a very potent and selective inhibitor of DPP8/9 with excellent membrane penetration ability, it could be used to probe their biological function in vivo using chemical biological methods. Further investigations of the biological functions of DPP8 and DPP9 with a knockout mouse study will help to shed light on their roles in cell physiology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.03.032.

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