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Discovery and pharmacological characterization of *N*-[2-({2-[(2*S*)-2-cyanopyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]-2-methylpyrazolo[1,5-*a*]pyrimidine-6-carboxamide hydrochloride (anagliptin hydrochloride salt) as a potent and selective DPP-IV inhibitor

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ABSTRACT

In the course of our program for discovery of novel DPP-IV inhibitors, a series of pyrazolo[1,5-*a*]pyrimidines were found to be novel DPP-IV inhibitors. We identified *N*-[2-({2-[(2*S*)-2-cyanopyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]-2-methylpyrazolo[1,5-*a*]pyrimidine-6-carboxamide hydrochloride (**4a**) and described its pharmacological profiles.

1. Introduction

Glucagon-like peptide-1 (GLP-1), a 30-amino acid peptide hormone, is secreted by intestinal L-cells in response to food ingestion and stimulates insulin secretion from β-cells in a glucose-dependent manner.[1] GLP-1 is also known to have multiple actions such as suppression of glucagon secretion, inhibition of gastric emptying and induction of satiety.[1,2] Based on these findings, GLP-1 has been considered to be an attractive target for the therapy of type 2 diabetes mellitus (T2DM). However, GLP-1 is rapidly degraded into inactive GLP-1 by a serine protease, dipeptidyl peptidase IV (DPP-IV), which fueled the development of biologically stable GLP-1 analogs.[3] Therefore, inhibitors of DPP-IV capable of increasing the circulating concentration of active GLP-1, have now emerged as promising treatments for T2DM.[4] In addition, it was demonstrated in a clinical study of diabetic patients receiving active GLP-1 infusion that a 24-h infusion of active GLP-1 resulted in a more marked improvement in glycemic control than a 16-h infusion,[5] and based on accumulating clinical studies, greater than 2-fold enhancement of circulating levels of active GLP-1 is known to result from inhibition of 80% or more of the plasma DPP-IV activity.[6] Consequently, optimal glycemic control requires continuous high-level exposure to DPP-IV inhibitors.

Following approval of sitagliptin (Januvia)[7] in the US in 2006, vildagliptin,[8] saxagliptin[9] and alogliptin[10] have been launched or have been under late stage clinical development (Fig. 1). Their emerging medical need was also exemplified by the fact that the combined sales of Januvia and Janumet[11] (sitagliptin/metformin) have been growing at double-digit rates in all regions and accounted for an 8% market share 3 years after their introduction into the US and Asian oral anti-diabetic markets.[12] Their rapid and wide acceptance is thought to be due to a low incidence of hypoglycemia and absence of body weight gain as well as excellent tolerability. DPP-IV inhibitors on the market are structurally diverse and therefore the expected growth in prescriptions for DPP-IV inhibitors would underscore compound-specific differences in efficacy and side effects. Thus, there seem to be opportunities for development of new chemical entities that have unique pharmacological profiles and/or different pharmacokinetic properties, which include the metabolic pathways.[13]

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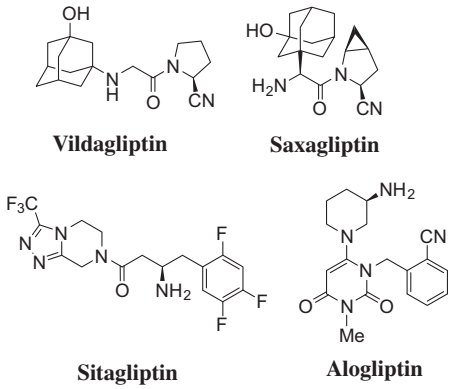


Figure 1. Some gliptins.

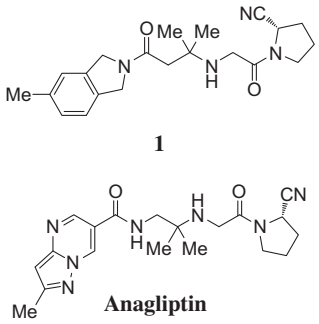
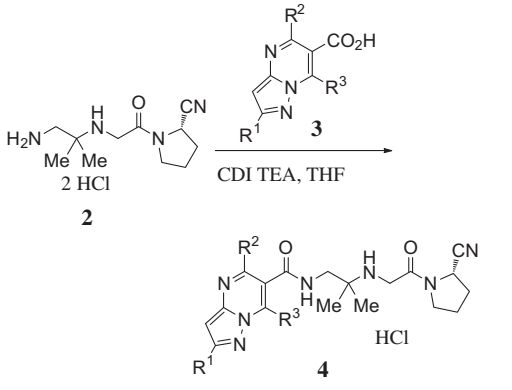


Figure 2. Anagliptin and its prototype **1**.

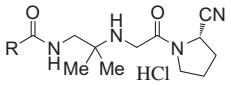


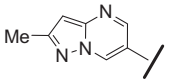
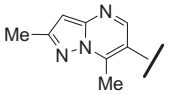
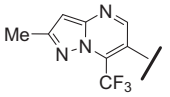
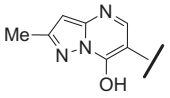
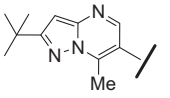
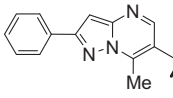
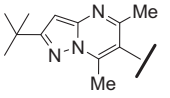
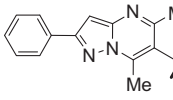
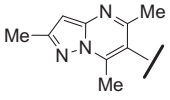
a: R^1 = Me, R^2 = R^3 = H
b: R^1 = Me, R^2 = H, R^3 = Me
c: R^1 = Me, R^2 = H, R^3 = CF_3
d: R^1 = Me, R^2 = H, R^3 = OH
e: R^1 = CMe_3, R^2 = H, R^3 = Me
f: R^1 = Ph, R^2 = H, R^3 = Me
g: R^1 = CMe_3, R^2 = R^3 = Me
h: R^1 = Ph, R^2 = R^3 = Me
i: R^1 = R^2 = R^3 = Me

Scheme 1. Synthesis of compounds **4**.

As part of our effort to discover novel DPP-IV inhibitors, we previously reported that SARs of isoindoline derivatives led to the discovery of 2-[3-[2-{(2*S*)-cyanopyrrolidin-1-yl}-2-oxoethylamino]-3-methyl-1-oxobutyl]-5-methyl-1,3-dihydroisoindole **1** as a highly selective, potent DPP-IV inhibitor.[14] Unfortunately, in vivo studies showed that an active metabolite of **1** had a very high inhibitory potency toward DPP-IV and we abandoned further development of the compounds in this series. Further studies showed that pyrazolo[1,5-*a*]pyrimidine functioned as a bioisostere of the isoindoline moiety that imparted improved metabolic stability

Table 1
DPP inhibitory activities of **4**[a]



Compound **4**	R	IC_{50}		
		DPP-IV (nM)	DPP-8 (nM)	DPP-9 (nM)
a		3.8	68	60
b		13	76	64
c		32	>100	81
d		1.8	58	45
e		15	>100	66
f		11	43	30
g		80	>100	53
h		28	12	21
i		110	45	28

[a] IC_{50} values are the average of at least two independent experiments.

and safety. Herein, we would like to report the synthesis and pharmacological profiles of novel and potent DPP-IV inhibitors, the hydrochloride salts of *N*-[2-({2-[(2*S*)-2-cyanopyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]-2-methylpyrazolo[1,5-*a*]pyrimidine-6-carboxamide (anagliptin)[15] and related compounds (Fig. 2).

2. Chemistry

A series of pyrazolo[1,5-*a*]pyrimidine derivatives were prepared as described in Scheme 1. 2-Amino-2-methylpropylamine was selectively protected with di-*tert*-butyl dicarbonate and coupled with (*S*)-1-(2-chloroacetyl)pyrrolidine-2-carbonitrile. After deprotection of the Boc group, compound **2** was WSC-coupled with various pyrazolo[1,5-*a*]pyrimidinecarboxylic acids **3a**–**h** to yield the desired compounds **4a**–**h**. All unavailable acids were prepared by methods described in the literature.[14]

3. Results and discussion

As described previously,[14] compounds **4** were evaluated in vitro for inhibition of human recombinant DPP-IV and were also

Table 2
Selected PK parameters of 4a and 4b in rats and dogs

Compound	Species	CL_{tot} (l/h/kg)	V_{dss} (l/h/kg)	C_{max}[c] (ng/ml)	t_{max}[c] (h)	$t_{1/2}$ (h)	AUC (ng/h/ml)	BA (%)
		iv		po				
4a[a]	Rat	2.00	0.68	309 (62)	0.8 (2.3)	1.9	1160	23
	Dog	0.65	0.83	261	1.5	1.0	824	100
4b[b]	Rat	2.23	1.61	747 (68)	0.25 (0.25)	1.9	1111	27
	Dog	0.62	0.75	282	1.0	1.3	6390	98

[a] Compound **4a** dose in rats, 1 mg/kg, iv (n = 3); 10 mg/kg, po (n = 3). **4a** dose in dogs, 0.2 mg/kg, iv (n = 3); 0.5 mg/kg, po (n = 2).
[b] Compound **4b** dose in rats, 3 mg/kg, iv (n = 1); 10 mg/kg, po (n = 1). **4b** dose in dogs, 0.2 mg/kg, iv (n = 2); 0.5 mg/kg, po (n = 2). CL_{tot}, plasma clearance; V_{dss}, steady-state volume of distribution; C_{max}, maximal concentration; t_{max}, time of maximal concentration; $t_{1/2}$, terminal half-life; AUC, area under the curve of plasma concentration; BA, oral bioavailability. Data are given as the mean value for the indicated compound (compd; **4a** or **4b**), species (rat or dog), dose (see above), and route of administration (iv or po).
[c] Values in parentheses were obtained at a **4a** or **4b** dose of 3 mg/kg (**4a**, n = 3; **4b**, n = 1).

Table 3
Serum protein binding of **4a** and **4b** in different species

Compd	Species	Protein binding (%)	
		20 hg/ml[a]	2000 hg/ml[a]
4a	Rat	77.3	77.1
	Dog	64.8	51.8
	Human	29.2	26.4
4b	Rat	22.1	17.9
	Dog	22.5	19.9
	Human	18.9	21.6

[a] Final compound concentration. Each value represents the mean of three experiments.

screened for selectivity over dipeptidyl peptidase 8 and 9 (DPP-8/9) by a fluorescence assay using glycyl-proline 4-methylcoumaryl-7-amide (Gly-Pro-MCA). Structure–activity relationships for compounds in this series are summarized in Table 1. DPP-8/9 data are also presented because the importance of selectivity over DPP-8/9 has been frequently mentioned in animal toxicity studies.[4] Our parent compound in this series, the 2-methyl pyrazolopyrimidine analogues **4a**, had DPP-IV inhibitory activity at an IC_{50} of 3.8 nM with more than 10,000-fold selectivity over DPP-8/9.[16] Substitution at the 7-position of the fused ring (e.g., **4b**–**d**) was well tolerated and the hydroxyl analogue **4d** was the most potent with an IC_{50} of 1.8 nM and excellent selectivity over DPP-8/9. Like the 7-substituents, replacement of the methyl group with bulkier alkyl groups such as the *tert*-butyl or phenyl group yielded compounds **4e** and **4f**, which had similar DPP-IV inhibitory potencies compared with **4b**. On the other hand, introduction of a methyl group at the 5-position (e.g., **4g**–**i**) resulted in slight to significant loss of the inhibitory activity relative to the corresponding unsubstituted analogues **4b**, **4e** and **4f**.

Eventually, taking their selectivity into account, compounds **4a**, **4b** and **4d** were chosen for further investigation. However, early pharmacokinetic (PK) studies showed compound **4d** to have a very

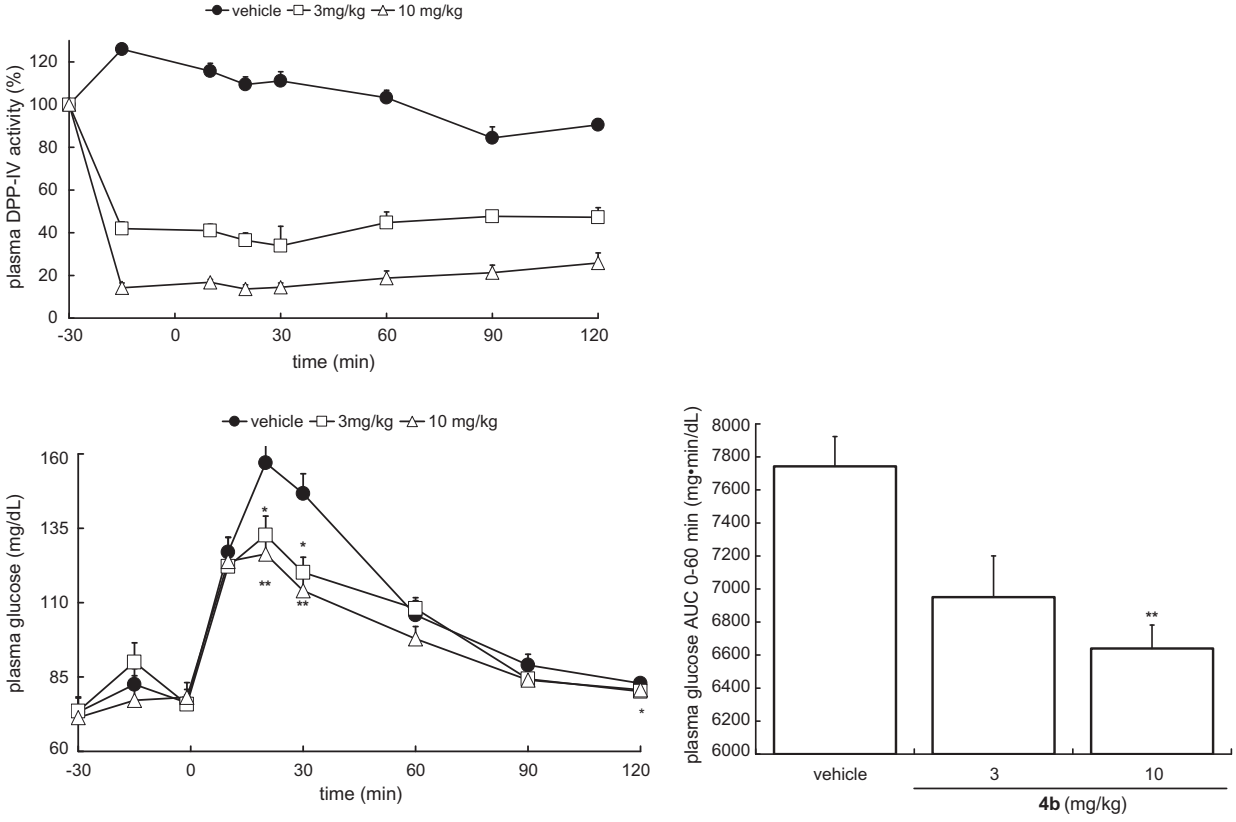


Figure 3. Effect of **4b** on plasma DPP-IV activity (upper graph) and plasma glucose concentration (lower left) after oGTT in rats; control group received vehicle after oGTT. Glucose AUC (lower right) was determined between 0 and 60 min. Asterisks in lower plots indicate significance at p <0.05 versus control (*) and p <0.01 versus control (**), respectively, by Dunnett's test.

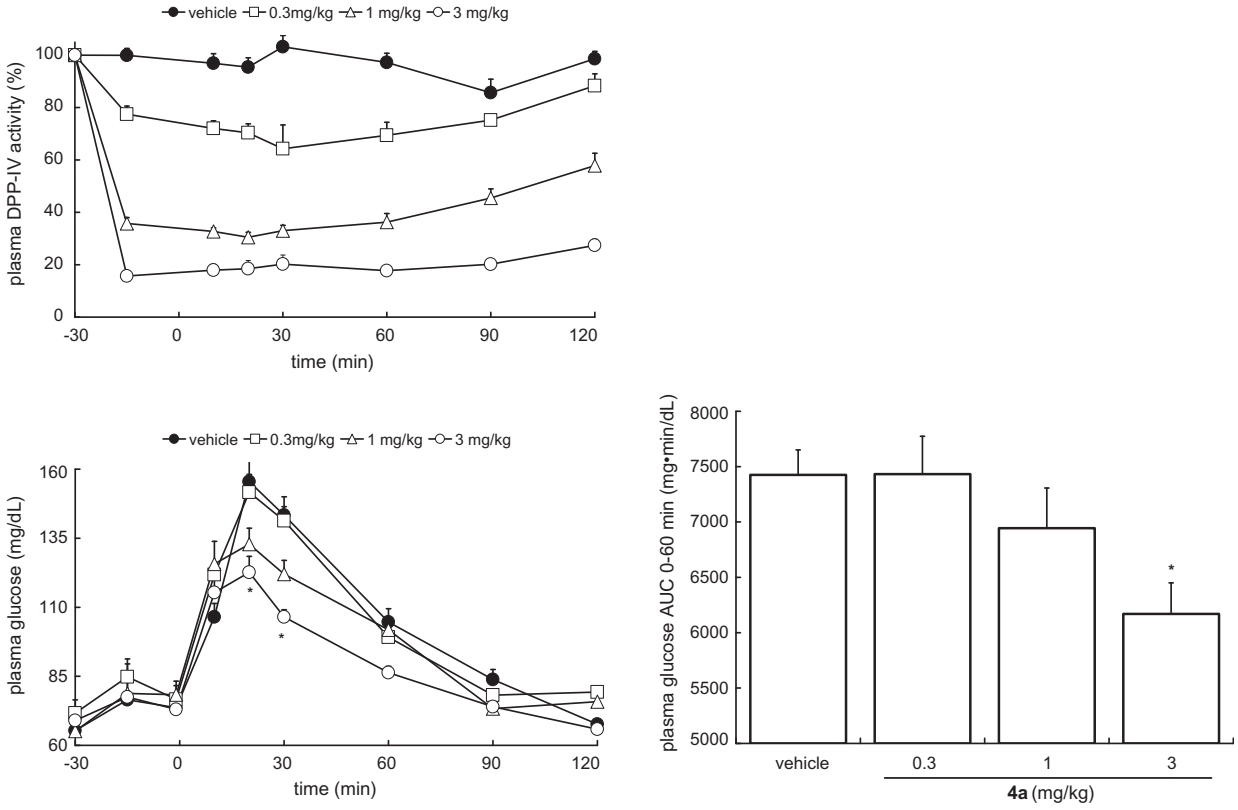


Figure 4. Effect of **4a** on plasma DPP-IV activity (upper graph) and plasma glucose concentration (lower left) after oGTT in rats; control group received vehicle after oGTT. Glucose AUC (lower right) was determined between 0 and 60 min. In lower plots, asterisk (*) indicates significance (p <0.05) versus control by Dunnett's test.

low oral bioavailability of 4.1% due to poor systemic exposure at a dose of 5 mg/kg po in Sprague–Dawley rats and then characterization of **4d** was discontinued. Selected PK parameters of **4a** and **4b** are listed in Table 2. These two compounds had a low clearance (**4a**, 0.65 L/h/kg; **4b**, 0.62 L/h/kg) and very high oral bioavailability (**4a**, 100%; **4b**, 98%) in beagle dogs, while showing a relatively high clearance (**4a**, 2.00 L/h/kg; **4b**, 2.23 L/h/kg) and moderate oral bioavailability in rats. As has been recently reported,[17] high levels of plasma protein binding of the inhibitor are considered a cause for the disconnection between the observed in vivo efficacy and the measured in vitro potency. To see if this would be the case with our compounds, their plasma protein binding was measured Table 3. The measured binding activity was reasonably acceptable across species. Remarkably, both compounds showed the lowest protein binding (~20%) in human plasma and were expected to be suitable for animal scale-up studies.

Given the preliminary PK data, we examined the potencies of **4a** and **4b** in oral glucose tolerance tests (oGTT). Fasted male Wistar/ST rats received orally administered vehicle, or compound **4a** or **4b** at different dosages. Half an hour after treatment (t = 0), oral glucose challenges (1 g/kg) were conducted. Plasma DPP-IV activities and plasma glucose levels were then monitored at various intervals over a 2 h period. Selected data are shown in Figures 3 and 4. At a dose of 10 mg/kg, **4b** caused approximately 90% inhibition of plasma DPP-IV activity within 30 min, and the high levels of inhibition were sustained throughout the study. The inhibitory effect was dose-dependent, with a 3 mg/kg dose yielding approximately 60% inhibition. Hence, reduction of the rise in blood glucose in response to glucose challenge paralleled DPP-IV inhibition, and a significant reduction of $AUC_{0-60\ min}$ was observed at the 10 mg/kg dose. More strikingly, administration of compound **4a** at a dose of 3 mg/kg gave 95% inhibition of plasma DPP-IV activity and produced greater than 90% inhibition throughout the study. The inhibitory effect was also dose-dependent, with a dose of only 0.1 mg/kg yielding 30% inhibition. Likewise, a significant reduction in the AUC was observed at the 1 mg/kg dose. In addition, increased insulin levels at 10 min post-challenge strongly suggested preservation of active GLP-1 (data not shown). The results showed a strong correlation between in vivo levels of plasma DPP-IV inhibition and in vivo efficacy, and confirmed superiority of **4a** over **4b** in improvement of glucose tolerance.[18]

Besides evaluation of its potential for anti-diabetics, compound **4a** was found to show an excellent preclinical safety profile; it did not act as inhibitor or inducer of major liver metabolic enzymes such as CYP3A4, CYP2C19, CYP2C9, CYP1A2, and CYP2D6 (IC_{50} >10 μM) or CYP3A4, CYP2C8, CYP2C9, and CYP1A2 at a concentration of 50 μM. Compound **4a** showed no binding to the hERG channel (E-4031, >90% inhibition at 0.1 μM, IC_{50} >500 μM in patch clamp using HEK 293 cells). **4a** was negative in the Ames mutagenicity test. It was also tested against a panel of 62 receptor binding/ion channel assays at a concentration of 10 μM, and did not show specific binding of greater than 20% in any of the 62 assays under control conditions.[19] Briefly, preliminary studies on the excretion pathway were conducted (Table 4). In bile duct-cannulated rats, 30% and 40% of **4a** were excreted unchanged from the urine in the 24 h period after iv administration at doses of 1 and 5 mg/kg, respectively.[20] Biliary excretion was also an important elimination pathway, with recovery of ~13% to 14% of the doses in bile. Similarly, in dogs dosed at 1 mg/kg iv, 32% and 24% of **4a** were excreted unchanged from urine and feces, respectively, in the 24 h period after drug administration. Parent compound **4a** was primarily eliminated into urine and biliary excretion was more significant in dogs than in rats. The approximately 50% recoveries of **4a** in these species were consistent with the data on in vitro metabolism,

Table 4
Excertion of **4a** after iv administration and metabolic stability

Excretion					Metabolic activity	
		Recovery of **4a** (% dose)[a]				
Species	Dose (mg/kg)	Urine	Bile	Feces	CL'_{int} (l/h/kg)	
Rat	1	30	13	ND	Rat	1.4
	5	41	14	ND	Dog	0.1
Dog	1	32	ND	24	Monkey	0.5
					Human	0.3

[a] Excretion of **4a** in bile duct-cannulated rats ($n = 2$) and dogs ($n = 3$) after iv administration. ND; not determined.

wherein the stability of **4a** in rodent and human liver microsomes was moderate to excellent (Table 4). Taken together, the data suggest that compound **4a** has a well-balanced elimination route.

4. Conclusions

In the course of our program for discovery of novel DPP-IV inhibitors, a series of pyrazolo[1,5-*a*]pyrimidines were found to be novel DPP-IV inhibitors. We identified *N*-[2-({2-[(2*S*)-2-cyanopyrrolidin-1-yl]-2-oxoethyl}amino)-2-methylpropyl]-2-methylpyrazolo[1,5-*a*]pyrimidine-6-carboxamide hydrochloride (**4a**) as a potent and selective DPP-IV inhibitor and have demonstrated several unique pharmacological profiles. On the basis of the data presented above, anagliptin, a free base of **4a**, was chosen for further evaluation as an option for DPP-IV inhibitors. Currently, late stage development is under way.

5. Experimental

5.1. Compound synthesis

General: All commercially available reagents and solvents were used without further purification. All reactions were carried out using oven-dried flasks or glassware, and the mixtures were stirred with magnetic stirring bars and concentrated using a standard rotary evaporator, unless otherwise noted. Procedures for preparation of all intermediates **2** and **3a**–**h** were described previously[14a] and can be found in the supplementary data. The ^{1}H NMR spectra were recorded by a JEOL JNM-ECP400 spectrometer operating at 400 MHz in DMSO-d_6 at 25 °C with tetramethylsilane as the internal standard. The data are reported as follows: chemical shift in ppm (δ), integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad singlet, m = multiplet), and coupling constant (Hz). LC/MS spectra were determined on a Waters ZMD2000 equipped with a Waters 2690 injector and a PDA detector operating at 210–400 nm and interfaced with a Micromass ZMD mass spectrometer. Infrared spectra (IR) were recorded on a JASCO FT/IR-4200 spectrometer. High-resolution mass spectra (HRMS) were recorded on a Thermo. LTQ Orbitrap.

5.1.1. Representative procedure for compound 4; (*S*)-2,7-Dimethylpyrazolo[1,5-*a*]pyrimidine-6-carboxylic acid {2-[(2-cyanopyrrolidin-1-yl)-2-oxoethylamino]-2-methylpropyl} amide hydrochloride (4b)

N,*N*′-Carbonyl diimidazole (930 mg) was added to a solution of **3b** (1.00 g) in tetrahydrofuran (30 ml), and the mixture was stirred at room temperature for 4 h. The reaction mixture was slowly added dropwise to **2** (1.56 g) in an ice-cooled solution of triethylamine (3.6 ml) in tetrahydrofuran (30 ml). The mixture was returned to room temperature and stirred overnight. The reaction mixture was concentrated under reduced pressure, and then dichloromethane was added to the residues. Insoluble materials were removed by filtration and the filtrate was concentrated under reduced pressure. The residues were subjected to column chromatography (eluting solvent; dichloromethane/methanol, 50:1) to yield a free base of **4b** (690 mg, 33%). 4 N hydrochloric acid/1,4-dioxane (0.50 ml) was added at 10 °C to a solution of the resulting compound (690 mg) in 1,4-dioxane (5.0 ml) and stirred for 10 min. Crystals were precipitated by adding ether and were then collected by filtration. The crystals were dried under reduced pressure to give **4b** (670 mg, 90%) as yellow crystals.

^{1}H NMR: δ 1.37 (6H, s), 2.05–2.31 (4H, m), 2.47 (3H, s), 2.87 (3H, s), 3.30–3.80 (4H, m), 4.10–4.30 (2H, m), 4.84–4.86 (1H, m), 6.60 (1H, s), 8.68 (1H, s), 8.93–8.97 (3H, m); MS *m/z* 398 $(M+H)^+$; IR (ATR) 2974, 2348, 2138, 1981, 1647, 1604, 1523, 1433, 1308, 1173, 422 cm^{-1}; HRMS calcd for $C_{20}H_{28}N_7O_2$: 398.2299. Found: 398.2305.

5.1.2. (*S*)-2-Methylpyrazolo[1,5-*a*]pyrimidine-6-carboxylic acid {2-[(2-cyanopyrrolidin-1-yl)-2-oxoethylamino]-2-methylpropyl} amide hydrochloride (4a)

^{1}H NMR: δ 1.36 (6H, s), 2.00–2.30 (4H, m), 2.50 (3H, s), 3.30–3.80 (4H, m), 4.10–4.30 (2H, m), 4.80 (1H, m), 6.63 (1H, s), 8.80–8.90 (3H, m), 9.50 (1H, s); MS *m/z* 384 $(M+H)^+$; Anal. Calcd for $C_{19}H_{25}N_7O_2{\cdot}HCl$: C, 59.51; H, 6.57; N, 25.57. Found: C, 59.91; H, 6.35; N, 25.71; IR (ATR) 3064, 2074, 2029, 1646, 1532, 1406, 1332, 1297, 1176, 665, 472, 413 cm^{-1}; HRMS calcd for $C_{19}H_{26}N_7O_2$: 384.2142. Found: 384.2147.

5.1.3. (*S*)-2-Methyl-7-trifluoromethylpyrazolo[1,5-*a*]pyrimidine-6-carboxylic acid{2-[(2-cyanopyrrolidin-1-yl)-2-oxoethylamino]-2-methylpropyl}amide hydrochloride (4c)

^{1}H NMR: δ 1.37 (6H, s), 2.02–2.10 (2H, m), 2.19–2.24 (2H, m), 2.53 (3H, s), 3.49–3.62 (3H, m), 3.72 (1H, m), 4.13–4.16 (2H, m), 4.86 (1H, dd, $J = 4.4$, 6.9 Hz), 6.94 (1H, s), 9.00 (2H, br s), 9.09 (1H, t, $J = 6.2$ Hz), 9.77 (1H, s); MS *m/z* 452 $(M+H)^+$.

5.1.4. (*S*)-2-Methyl-7-hydroxypyrazolo[1,5-*a*]pyrimidine-6-carboxylic acid{2-[(2-cyanopyrrolidin-1-yl)-2-oxoethylamino]-2-methylpropyl}amide hydrochloride (4d)

^{1}H NMR: δ 1.22 (6H, s), 2.05–2.27 (4H, m), 2.28 (3H, s), 3.48–3.53 (4H, m), 3.65 (1H, m), 3.79–3.89 (2H, m), 4.79–4.82 (1H, m), 5.97 (1H, s), 8.47 (1H, s), 9.65 (1H, br t); MS *m/z* 400 $(M+H)^+$; IR (ATR) 3746, 3277, 2353, 2239, 2182, 2042, 1637, 1527, 1418, 1305, 1180, 792, 469, 418 cm^{-1}; HRMS calcd for $C_{19}H_{26}N_7O_3$: 400.2091. Found: 400.2096.

5.1.5. (*S*)-2-*tert*-Butyl-7-methylpyrazolo[1,5-*a*]pyrimidine-6-carboxylic acid{2-[(2-cyanopyrrolidin-1-yl)-2-oxoethylamino]-2-methylpropyl}amide hydrochloride (4e)

^{1}H NMR: δ 1.36 (6H, s), 1.38 (9H, s), 1.97–2.08 (2H, m), 2.19–2.25 (2H, m), 2.88 (3H, s), 3.51–3.58 (3H, m), 3.73 (1H, m), 4.12–4.17 (2H, m), 4.87 (1H, dd, $J = 4.4$, 7.0 Hz), 6.73 (1H, s), 8.68 (1H, s), 8.91 (1H, t, $J = 6.2$ Hz), 8.95 (2H, br s); MS *m/z* 440 $(M+H)^+$; IR (ATR) 2961, 2361, 2325, 2135, 2035, 2007, 1661, 1603, 1523, 1434, 1311, 1215, 474, 418 cm^{-1}; HRMS calcd for $C_{23}H_{34}N_7O_2$: 440.2768. Found: 440.2770.

5.1.6. (*S*)-2-Phenyl-7-methylpyrazolo[1,5-*a*]pyrimidine-6-carboxylic acid{2-[(2-cyanopyrrolidin-1-yl)-2-oxoethylamino]-2-methylpropyl}amide hydrochloride (4f)

^{1}H NMR: δ 1.39 (6H, s), 2.03–2.11 (2H, m), 2.19–2.25 (2H, m), 2.96 (3H, s), 3.54–3.69 (4H, m), 4.11–4.23 (2H, m), 4.88 (1H, dd, $J = 4.0$, 7.0 Hz), 7.36 (1H, s), 7.47 (1H, t, $J = 7.3$ Hz), 7.52 (2H, dd, $J = 7.3$, 7.7 Hz), 8.10 (2H, d, $J = 7.7$ Hz), 8.77 (1H, s), 9.01–9.06 (3H, m); MS *m/z* 460 $(M+H)^+$; IR (ATR) 2979, 2329, 2116, 1994, 1660, 1604, 1579, 1524, 1460, 1436, 1400, 1314, 1260, 1174, 768, 693, 630, 419, 409 cm^{-1}; HRMS calcd for $C_{25}H_{30}N_7O_2$: 460.2455. Found: 460.2455.

5.1.7. (*S*)-2-*tert*-Butyl-5,7-dimethylpyrazolo[1,5-*a*]pyrimidine-6-carboxylic acid{2-[(2-cyanopyrrolidin-1-yl)-2-oxoethylamino]-2-methylpropyl}amide hydrochloride (4g)

^{1}H NMR: δ 1.36 (15H, s), 2.01–2.10 (2H, m), 2.19–2.24 (2H, m), 2.47 (3H, s), 2.66 (3H, s), 3.51–3.71 (4H, m), 4.11–4.19 (2H, m), 4.87 (1H, dd, J = 4.4, 6.6 Hz), 6.52 (1H, s), 8.80 (1H, t, J = 6.3 Hz), 9.08 (2H, br s); MS m/z 454 $(M+H)^+$; IR (ATR) 2964, 2361, 2333, 2070, 2010, 1656, 1539, 1432, 1281, 489 cm^{-1}; HRMS calcd for $C_{24}H_{36}N_7O_2$: 454.2925. Found: 454.2926.

5.1.8. (*S*)-2-Phenyl-5,7-dimethylpyrazolo[1,5-*a*]pyrimidine-6-carboxylic acid{2-[(2-cyanopyrrolidin-1-yl)-2-oxoethylamino]-2-methylpropyl}amide hydrochloride (4h)

^{1}H NMR: δ 1.38 (6H, s), 2.02–2.10 (2H, m), 2.19–2.25 (2H, m), 2.52 (3H, s), 2.75 (3H, s), 3.53–3.76 (4H, m), 4.11 (1H, dd, J = 6.6, 16.5 Hz), 4.18 (1H, dd, J = 5.9, 16.5 Hz), 4.87 (1H, dd, J = 4.4, 7.0 Hz), 7.18 (1H,s), 7.44 (1H, t, J = 7.3 Hz), 7.51 (2H, dd, J = 7.0, 7.3 Hz), 8.07 (2H, d, J = 7.0 Hz), 8.94 (1H, t, J = 6.0 Hz), 9.10 (2H, br s); MS m/z 474 $(M+H)^+$; IR (ATR) 2985, 2332, 2164, 1656, 1614, 1518, 1438, 1276, 1194, 769, 695, 445, 410 cm^{-1}; HRMS calcd for $C_{26}H_{32}N_7O_2$: 474.2612. Found: 474.2612.

5.1.9. (*S*)-2,5,7-Trimethylpyrazolo[1,5-*a*]pyrimidine-6-carboxylic acid{2-[(2-cyanopyrrolidin-1-yl)-2-oxoethylamino]-2-methylpropyl}amide hydrochloride (4i)

^{1}H NMR: δ 1.37 (6H, s), 1.98–2.09 (2H, m), 2.18–2.27 (2H, m), 2.43 (3H, s), 2.47 (3H, s), 2.66 (3H, s), 3.52–3.63 (1H, m), 3.62 (2H, d, J = 6.2 Hz), 3.71–3.76 (1H, m), 4.10–4.21 (2H, m), 4.86 (1H, dd, J = 4.4, 7.0 Hz), 6.44 (1H, s), 8.92 (1H, br t), 9.12 (2H, br s); MS m/z 412 $(M+H)^+$; IR (ATR) 2981, 2344, 2117, 1998, 1656, 1536, 1433, 1322, 1277, 1173, 731, 553, 434, 418 cm^{-1}; HRMS calcd for $C_{21}H_{30}N_7O_2$: 412.2455. Found: 412.2455.

5.2. Biological evaluation

General: Inhibition of DPP-IV and -8/9 activity and metabolic stability were determined as described previously[14b] and can be found in the supplementary data. All animal experiments were approved by the Animal Ethics Committee of the Sanwa kagaku kenkyusho.

5.2.1. Pharmacokinetic studies in rats

Male Sprague–Dawley rats (6–7 weeks old/160–180 g) were acclimated for at least 3 days before use. Food and water were supplied ad libitum at all times throughout the experiment. Compounds **4a** or **4b** were administered intravenously (iv) via the femoral vein (**4a**, 1 mg/kg, n = 3; **4b**, 3 mg/kg, n = 1) and by oral (po) gavage at a dose of 10 mg/kg in 5% gum arabic solution (**4a**; n = 3, **4b**; n = 1). Approximately 200 μl of blood was collected from the jugular vein with a heparinized syringe under diethyl ether anesthesia. Blood samples were collected at 0.083 (iv only), 0.167 (iv only), 0.25, 0.5, 1, 2, 3, 5, 7, 9, and 24 h post-dose. Plasma was obtained by centrifugation at 4 °C and stored at −70 °C until analysis. To determine the role of biliary excretion in the rat, two bile duct-cannulated rats received a single intravenous dose of **4a** (1 and 5 mg/kg). Urine and bile were collected at intervals of 0–8 and 8–24 h and stored at −70 °C until analysis.

5.2.2. Pharmacokinetic studies in Beagle dogs

This study was conducted to examine the pharmacokinetics and excretion of compounds **4a** and **4b** in a crossover experimental design. Three Beagle dogs (10–13 kg) were placed in individual stainless-steel metabolic cages. Animals were fasted overnight, before and 6 h after drug administration. The oral dose was prepared in hand-filled gelatin capsules. Dogs were given a single oral dose of **4a** or **4b** in the form of a capsule together with 50 ml of water (0.5 mg/kg, n = 2). Following a 1-week washout period, a single intravenous dose of **4a** or **4b** (0.2 mg/kg, n = 2) was given. Approximately 1.5 ml of blood was collected from the vein with a heparinized syringe at the following times: 0.083, 0.167, 0.25, 0.5, 1, 2, 3, 5, 7, 9, and 24 h for iv doses; 0.25, 0.5, 1, 2, 3, 5, 7, 9, and 24 h for oral doses. Plasma was obtained by centrifugation at 4 °C and stored at −70 °C until analysis. For excretion studies, three dogs were dosed intravenously with **4a** (1 mg/kg), and then urine was collected from the dogs at 0–7 and 7–24 h intervals. Fecal and cage wash samples were collected at 24 h post-dose.

5.2.3. Plasma pharmacokinetic analysis

Noncompartmental analysis was used to determine the pharmacokinetics of **4a** and **4b**. Maximal plasma concentration (C_{max}) and time of maximal plasma concentration (t_{max}) were assessed by visual inspection. The terminal elimination half-life was calculated using the relationship 0.693/k, where k is the elimination rate constant. The $AUC_{0\text{-}t}$ was calculated through the last measurable time point t by the trapezoidal rule. From the intravenous plasma concentration data, plasma clearance and volume of distribution of **4a** and **4b** were determined. BA was calculated according to the following equation:

$$BA(\%) = (AUC_{po}/D_{po})/(AUC_{iv}/D_{iv}) \times 100$$

where AUC_{po} is the AUC after oral dosing; AUC_{iv}, the AUC after intravenous dosing; D_{po}, the oral dose; and D_{iv}, the intravenous dose.

5.2.4. In vitro protein binding

Ultrafiltration units (VIVASPIN®, Sartorius) were used in protein binding studies. Rat serum, dog serum and human serum containing drug concentrations of 20 and 2000 ng/ml were centrifuged for 0.5 h at 37 °C incubation. Following centrifugation, the amount of drug in the supernatant was determined by LC–MS/MS. The fraction of bound to protein was calculated from the concentrations in the spiked sample and the supernatant.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.09.043.

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18. To rationalize the observed difference in in vivo potency of **4a** and **4b**, plasma concentrations of the two compounds available for DPP-IV inhibition (i.e., free fraction of concentration) were estimated. From the data listed in Tables 2 and 3, free fraction concentrations of **4a** and **4b** at the 3 mpk dose are calculated to be 14 and 54 nM at t_{max}, respectively, which suggest systemic exposures were sufficient for high levels (~90%) of in vivo inhibition in both cases. Our attempt at the rationalization failed, but it became evident that in vivo potency of **4a** was consistent with its in vitro potency.
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20. Pharmacokinetic studies with [^{14}C]compound **4a** in rats and dogs indicated that most (89–98%) of excretion occurred within 24 h. The detail will be reported elsewhere.