Original Article

Dipeptidyl Peptidase IV Inhibition for the Treatment of Type 2 Diabetes

Potential Importance of Selectivity Over Dipeptidyl Peptidases 8 and 9

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Dipeptidyl peptidase (DPP)-IV inhibitors are a new approach to the treatment of type 2 diabetes. DPP-IV is a member of a family of serine peptidases that includes quiescent cell proline dipeptidase (QPP), DPP8, and DPP9; DPP-IV is a key regulator of incretin hormones, but the functions of other family members are unknown. To determine the importance of selective DPP-IV inhibition for the treatment of diabetes, we tested selective inhibitors of DPP-IV, DPP8/DPP9, or QPP in 2-week rat toxicity studies and in acute dog tolerability studies. In rats, the DPP8/9 inhibitor produced alopecia, thrombocytopenia, reticulocytopenia, enlarged spleen, multiorgan histopathological changes, and mortality. In dogs, the DPP8/9 inhibitor produced gastrointestinal toxicity. The QPP inhibitor produced reticulocytopenia in rats only, and no toxicities were noted in either species for the selective DPP-IV inhibitor. The DPP8/9 inhibitor was also shown to attenuate T-cell activation in human in vitro models; a selective DPP-IV inhibitor was inactive in these assays. Moreover, we found DPP-IV inhibitors that were previously reported to be active in models of immune function to be more potent inhibitors of DPP8/9. These results suggest that assessment of selectivity of potential clinical candidates may be important to an optimal safety profile for this new class of antihyperglycemic agents. ***Diabetes*** **54:2988–2994, 2005**

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AMC, aminomethylcoumarin; DASH, DPP-IV activity and/or structure homologues; DPP, dipeptidyl peptidase; GLP-1, glucagon-like peptide 1; IL, interleukin; QPP, quiescent cell proline dipeptidase.

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Therapies that increase the circulating concentrations of insulin have proven beneficial in the treatment of type 2 diabetes. Dipeptidyl peptidase (DPP)-IV inhibitors are a promising new approach to type 2 diabetes that function, at least in part, as indirect stimulators of insulin secretion (1). Clinical proof of concept for the efficacy of DPP-IV inhibitors has been provided by LAF237 (2,3). Additional proof of concept has been obtained in studies with DPP-IV–deficient mice, which are healthy, fertile, and have improved metabolic function (4,5).

The efficacy of DPP-IV inhibitors is mediated primarily via stabilization of the incretin hormones glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide, which have clearly established roles in glucose-dependent insulin secretion (6). Subchronic (6 weeks) continuous infusion of GLP-1 resulted in profound and significant decreases in fasting plasma glucose and HbA_{1c} (1.3%) (7). GLP-1 is rapidly hydrolyzed ($t_{1/2}$ = ~1 min) in vivo to produce an inactive product, GLP-1[9–36] amide (8), and several lines of evidence indicate that DPP-IV, a proline-specific serine dipeptidyl aminopeptidase, is primarily responsible for this inactivation (9,10).

DPP-IV is the founding member of a family of DPP-IV activity and/or structure homologue (DASH) proteins, enzymes that are unified by their common postproline cleaving serine dipeptidyl peptidase mechanism (11). In addition to DPP-IV, family members include quiescent cell proline dipeptidase (QPP) (aka DPP7) (12), DPP8 (13), DPP9 (14), fibroblast activation protein (15), attractin (16), and DPP-IV-β (17). Except for DPP-IV, the functions of these enzymes are unknown. Nonetheless, based on their preference for cleavage after H_2N-X-Pro in vitro, they are likely to be involved in at least some of the increasing number of biological processes that appear to be regulated by proline-specific NH_2-terminal processing (18). Thus,

given the unknown consequences of inhibiting one or more of these family members, a potentially important consideration in the selection of DPP-IV inhibitors for clinical development is the degree of selectivity over other DASH family proteins required for an optimal safety profile.

DPP-IV is identical to CD26, a marker for activated T-cells (19). There have been several studies with Lys [Z(NO_2)] pyrrolidide and related compounds aimed at investigating the potential role of enzyme activity in immune function (20). These compounds have been shown to have several effects on immune cells, including inhibition of proliferation, cytokine production, and induction of transforming growth factor-β secretion. However, the finding that there are several DPP-IV homologs has prompted reexamination of these results, as the selectivity of these compounds has not been reported.

Herein, we describe the results of studies with broad-specificity DASH inhibitors and highly selective inhibitors of DPP-IV, QPP, and DPP8/9 in in vivo models for the assessment of preclinical safety and tolerability and in in vitro models of T-cell activation.

RESEARCH DESIGN AND METHODS

Compounds. The *threo*-2*S*,3*S*-isoleucyl thiazolidine furmarate (2:1) (compound 1) was provided by the Department of Process Research, Merck Research Laboratories (Rahway, NJ). The *allo*-2*S*,3*R*-isoleucyl thiazolidine fumarate (2:1) (compound 2) was purchased from Heumann Pharma (Nuernberg,Germany).The(2*R*)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo [4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,5-difluorophenyl)butan-2-amine fumarate and hydrochloride salts (compound 3), (2*S*,3*R*)-2-(2-amino-3-methyl-1-oxopentan-1-yl)-1,3-dihydro-2*H*-isoindole hydrochloride (compound 4), 1-[(2*S*)-2-amino-2-[(3*R*)-1-(4-iodophenyl)sulfonyl] pyrrolidin-3-yl]-1-oxoethyl]thiazolidine (compound 5), (2*S*)-2-[4-[[[[(2*S*)-1-[(3*R*)-3-amino-4-(2,5-difluorophenyl)-1-oxobutyl]-2-pyrrolidinyl]carbonyl]amino]methyl]phenoxy]-3-methylbutanoic acid, trifluoroacetate (compound 6), [(2*R*)-1-L-valylpyrrolidin-2-yl]boronic acid, trifluoroacetate (compound 7), 4-nitrobenzyl [(5*S*)-5-amino-6-oxo-6-(1-pyrrolidinyl)hexyl] carbamate (compound 8), 4-nitrobenzyl [(5*S*)-5-amino-6-oxo-6-(1,3-thiazolidin-3-yl)hexyl]carbamate (compound 9), and 4-nitrobenzyl [(5*S*)-5-amino-6-oxo-6-(1-piperidinyl)hexyl]carbamate (compound 10) were provided by the Department of Medicinal Chemistry, Merck Research Laboratories (Rahway, NJ).

Pharmacokinetics of selective inhibitors. The pharmacokinetics of compounds 1 and 2 in rats and dogs were previously reported (21). Similar study protocols and methods of analyses were used to evaluate the pharmacokinetics of compounds 3, 4, and 5.

In vitro assays

DPP-IV. To measure the activity of DPP-IV, a continuous fluorometric assay was employed using Gly-Pro-AMC, which is cleaved by the enzyme to release the fluorescent aminomethylcoumarin (AMC). A typical reaction contained 50 pmol/l enzyme, 50 μmol/l Gly-Pro-AMC, and buffer (100 mmol/l HEPES, pH 7.5, 0.1 mg/ml BSA) in a total reaction volume of 100 μl. Liberation of AMC was monitored using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The enzyme used in these studies was soluble human protein produced in a baculovirus expression system (Bac-To-Bac; Life Technologies).

DPP8. Compounds were tested against human DPP8 (baculovirus) in a continuous fluorescent assay in 50 mmol/l sodium phospate buffer, pH 8.0, and 0.1 mg/ml BSA, using Ala-Pro-7-amino-4-trifluormethylcoumarin as substrate at 100 μmol/l at 37°C for 15 min (excitation/emission: 400/505 nm).

DPP9. Compounds were tested against human DPP9 (baculovirus) in a continuous fluorescent assay in 100 mmol/l Tris/HCl buffer, pH 7.4, and 0.1 mg/ml BSA, using Gly-Pro-AMC as substrate at 100 μmol/l at 37°C for 30 min (excitation/emission: 360/460 nm).

QPP. Compounds were tested against human QPP (baculovirus) in a continuous fluorescent assay in 100 mmol/l cacodylate buffer, pH 5.5, and 0.1 mg/ml BSA, using Nle-Pro-AMC as substrate at 5 μmol/l at 37°C for 15 min (excitation/emission: 360/460 nm).

Fibroblast activation protein. Compounds were tested against human fibroblast activation protein ($Glu_{337} \rightarrow$ Gly mutant) (baculovirus) in a continuous fluorescent assay in 100 mmol/l Tris/HCl buffer, pH 8.0, 150 mmol/l NaCl, and 0.1 mg/ml BSA, using Nle-Pro-AMC as substrate at 50 μmol/l at 37°C for 15 min (excitation/emission: 360/460 nm).

Aminopeptidase P. Compounds were tested against human aminopeptidase P (*E. coli*) in a continuous fluorescent assay in 40 mmol/l Tris/HCl buffer, pH 7.4, 0.1 mg/ml BSA, 50 mmol/l NaCl, and 1 mmol/l $MnCl_2$, using Lys(Abz)Pro-Pro-pNA as substrate at 40 μmol/l at 37°C for 30 min (excitation/emission: 310/410 nm).

Prolyl endopeptidase. Compounds were tested against human prolyl endopeptidase (*E.coli*) in a continuous fluorescent assay in 100 mmol/l sodium phospate buffer, pH 7.5, 0.1 mg/ml BSA, 1 mmol/l EDTA, 5 mmol/l fresh dithiothreitol, and 100 mmol/l NaCl, using Z-Gly-Pro-AMC as substrate at 50 μmol/l at 37°C for 30 min (excitation/emission: 360/460 nm).

Prolidase. Compounds were tested against prolidase from porcine kidney (Biozyme Laboratories) in a continuous UV/Vis assay in 50 mmol/l Tris/HCl buffer, pH 8.0, using 10 mmol/l Gly-Pro as substrate at 10 mmol/l at 37°C for 15 min (absorption monitored at 230 nm).

Data analysis. To measure the inhibition constants, serial dilutions of inhibitor were added to reactions containing enzyme and substrate. IC_{50} values were determined by a fit of the reaction rates to a three-parameter Hill equation by nonlinear regression. To determine the dissociation constants (K_i), reaction rates were fit by nonlinear regression to the Michaelis-Menton equation for competitive, reversible inhibition.

In vitro assays for T-cell activation. Human peripheral blood mononuclear cells were isolated from healthy volunteers and stimulated overnight with either 10 μg/ml phytohemagglutinin or with a mixture containing 100 pg/ml each of staphylococcal enterotoxins A, B, C1, D, and E (Toxin Technologies). Interleukin (IL)-2 concentrations in the culture supernatant were measured by immunoassay. Cell proliferation was measured by addition of 1 μCi ^{3}H-thymidine followed by an overnight culture.

Oral glucose tolerance test in diet-induced obese mice. Male C57BL/6 mice (6–8 weeks of age) were obtained from Taconic Farms (Germantown, NY). Mice were fed a lean (control group) or high-fat (DIO group) diet (5 and 35% fat by weight, respectively) for 6–9 weeks and then administered an oral glucose tolerance test. Fasted DIO mice were orally dosed with vehicle (0.5% methylcellulose), compound 1, or compound 2 at 3, 10, or 30 mg/kg, 1 h before glucose (2 g/kg) challenge. Blood glucose was determined at various time points from tail bleeds using a glucometer.

Rat toxicity studies. Crl:CD(SD)IGS BR rats ~3–4 weeks of age were obtained from Charles River Laboratories, Wilmington, NC. At 6 weeks of age, rats (five rats per sex per group) were administered vehicle (0.5% methylcellulose) or compound (10, 30, and 100 $mg \cdot kg^{-1} \cdot day^{-1}$) by oral gavage (5 ml/kg). Animals were observed daily for physical signs of toxicity. During the 2nd week of dosing, all rats were anesthetized with isoflurane, and blood samples were taken for determination of complete blood counts. In addition, serum samples from fasted rats were analyzed for a complete panel of clinical chemistry parameters. Urine was collected overnight for routine urinalysis. At termination of the study, all rats were killed, and a complete necropsy was conducted. An extensive list of tissues were dissected from all rats, weighed, fixed in 10% neutral buffered formalin, and processed by routine histology methods for microscopic examination.

Acute dog tolerability studies. Purpose-bred Beagle dogs were obtained from Marshall Farms, North Rose, NY. All dogs were acclimated for at least 4 weeks before study initiation. All compounds were formulated as aqueous suspensions in 0.5% methylcellulose and orally administered via gavage at a dose volume of 5 ml/kg. Following oral dosing, all animals were observed for several hours at frequent intervals and clinical signs of toxicity recorded for each dog.

Two-week toxicity study conducted in DPP-IV–deficient mice. Male and female DPP-IV–deficient (4) and wild-type (C57BL/6) mice were obtained at 6 weeks of age from Taconic Farms and acclimated for 2 weeks before the initiation of dosing. DPP-IV–deficient mice (six mice per sex per group) were administered vehicle (0.25% methylcellulose) or the DPP8/9-selective inhibitor (30, 100, and 300 $mg \cdot kg^{-1} \cdot day^{-1}$) via oral gavage (5 ml/kg). Wild-type mice were administered compound at 300 $mg \cdot kg^{-1} \cdot day^{-1}$. Animals were observed daily for physical signs of toxicity. At the completion of the 2-week study, a terminal blood sample was collected by cardiac puncture for complete blood counts. A necropsy was conducted and liver and spleen organ weight were recorded. Selected tissues were fixed in 10% neutral buffered formalin and processed by routine histology methods for microscopic examination. Tissue sections from all control and treatment groups were evaluated.

All in vivo procedures described above were conducted in laboratories accredited by AAALAC (Association for the Assessment and Accreditation of Laboratory Animal Care) International. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Merck Research Laboratories.

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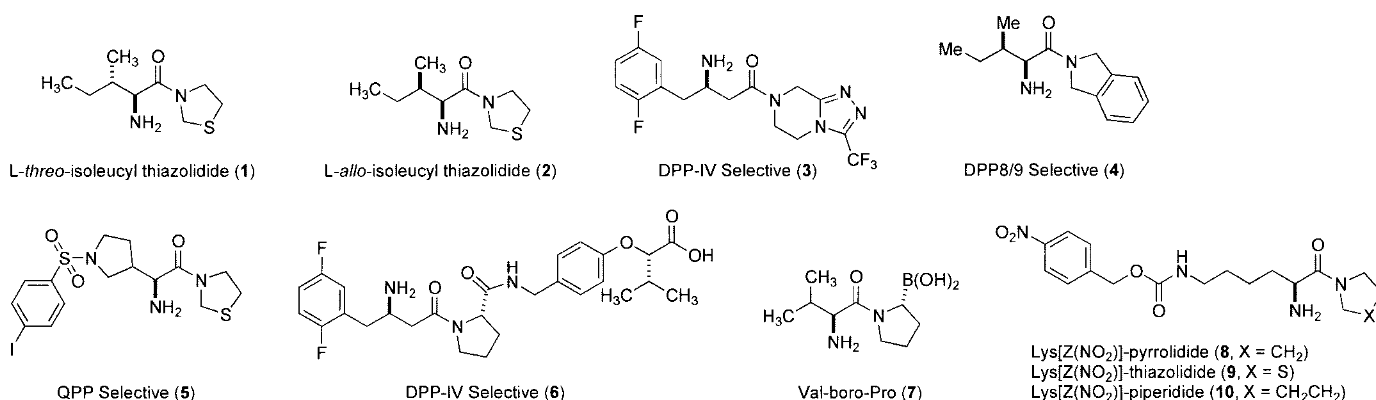

FIG. 1. Structures of compounds used in this study.

RESULTS

Preclinical toxicities of threo and allo isomers of isoleucyl thiazolidine. Several inhibitors of DPP-IV have been described (22). One representative example is L-*threo*-isoleucyl thiazolidine 1 (Fig. 1, compound 1) a DPP-IV inhibitor that has demonstrated efficacy in several animal models of diabetes (23–26). To evaluate the safety of this compound in preclinical species, 4-week toxicity studies in rats and dogs were conducted. In rats, toxicities were limited to the presence of lung histiocytosis and thrombocytopenia at relatively high doses (77.5 and 698 mg/kg, respectively). In dogs, acute central nervous system toxicities, characterized by ataxia, seizures, convulsions, and tremor, were observed at a dose of 75 mg/kg after one or two doses. Bloody diarrhea was observed at 225 mg/kg. No additional toxicities were observed upon dosing for 4 weeks. However, in subsequent chronic toxicity studies, upon 5–6 weeks of treatment with this compound in dogs, mortality and profound toxicities occurred at doses $\geq$25 mg $\cdot$ kg^{-1} $\cdot$ day^{-1}. These toxicities included anemia, thrombocytopenia, splenomegaly, and multiple organ pathology, mainly affecting the lymphoid system and gastrointestinal tract (Table 1).

The *allo* isomer of isoleucyl thiazolidine, compound 2, when compared with the *threo* isomer, has virtually identical affinity to DPP-IV (Fig. 2*A*), similar pharmacokinetic (Table 2) and metabolic profiles (21), and similar in vivo efficacy in an oral glucose tolerance test in diet-induced obese mice (Fig. 2*B*). This compound was evaluated in 4-week toxicity studies in rats and acute tolerability studies in dogs. Although the toxicity profiles of the two compounds are qualitatively similar in both rats and dogs, the *allo* isomer was ~10-fold more toxic when compared on either a dose level or plasma exposure basis (Table 1). In view of the comparable pharmacodynamic activity and pharmacokinetics in both species (Fig. 2 and Table 2), these results suggested that the toxicities were likely not due to DPP-IV inhibition but instead were potentially due to off-target activity. Evidence that these toxicities might be due to the inhibition of one or more proline-specific dipeptidyl peptidases was provided by studies with tissue extracts from DPP-IV–deficient mice. Detergent-solubilized extracts from the kidneys, liver, lung, and gastrointestinal tract of these animals were found to contain low levels of a proline-specific dipeptidyl peptidase activity, detected using the fluorogenic substrate Gly-Pro-AMC. The proline-selective dipeptidase activity in DPP-IV–deficient mice was 10- to 25-fold lower than that measured in corresponding tissues of wild-type animals and, unlike DPP-IV, was differentially inhibited by *threo* and *allo* isoleucyl thiazolidine (IC_{50} = 726 and 86 nmol/l, respectively). The 8.5-fold greater potency of the *allo* diastereomer against this activity suggested that off-target inhibition of one or more DPP-IV–like peptidases by this inhibitor could be responsible for preclinical toxicity.

TABLE 1
Preclinical toxicities of *allo*- and *threo*-isoleucyl thiazolidine

Species	Toxicity	*Threo* (mg/kg)	*Allo* (mg/kg)
Rat	Lung histiocytosis	77.5	10
	Thrombocytopenia	698	10
	Anemia	neg @ 698	100
	Splenomegaly/ lymphadenopathy	neg @ 698	10
	Mortality with multiple organ pathology	neg @ 698	300
Dog	Acute gastrointestinal toxicity	225	10
	Acute central nervous system toxicity	75	NT
	Thrombocytopenia	25	NT
	Anemia	25	NT
	Splenomegaly/ lymphadenopathy	25	NT
	Mortality with multiple organ pathology	75	NT

neg, negative finding at indicated dose; NT, not tested.

Selectivity of *threo*- and *allo*-isoleucyl thiazolidines. The selectivity of the *threo* and *allo* isomers was determined in activity assays against a panel of diverse proteases, including DPP-IV–related peptidases. The compounds were also screened by MDS Pharma Services (PanLabs) in a panel of 170 receptor and enzyme assays. No significant activity (IC_{50} <100 μmol/l) was observed in any of the PanLabs assays, with the exception of the sigma σ_1 receptor for the *allo* compound (K_i = 42 μmol/l). IC_{50s} >100 μmol/l were observed for inhibition of several other proteases (cathepsins B and H, HIV protease, caspases 1–3 and 13, granzyme B, chymotrypsin, β and γ secretases, thrombin, trypsin, factor Xa, plasmin, tissue plasminogen activator, activated protein C, plasma kallikrein, and urokinase). For DPP-IV–related dipeptidyl peptidases (Table 3), inhibition was only observed for DPP-IV and the related dipeptidyl peptidases, QPP, DPP8, and DPP9. Inhibition of DPP8 and QPP by isoleucyl thiazolidine has been previously reported (27). Comparable QPP inhibition

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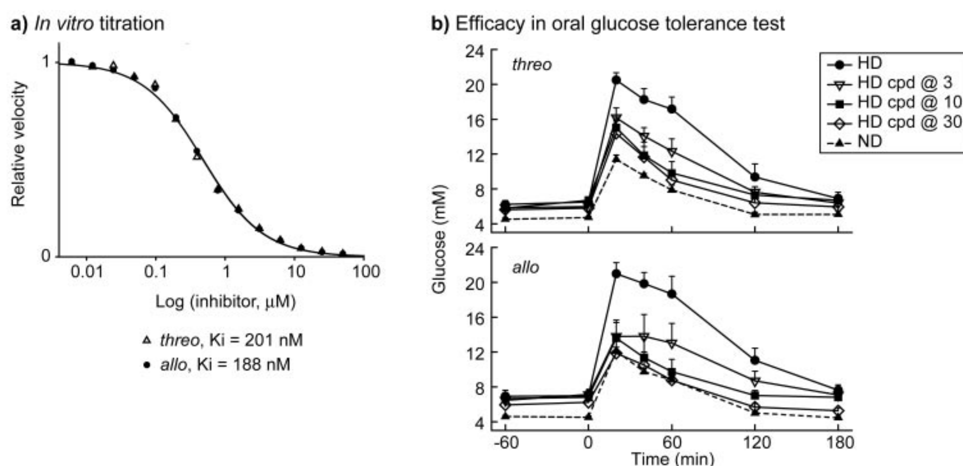

FIG. 2. In vitro and in vivo evaluation of *threo* and *allo* isomers of isoleucyl thiazolidine. *A*: *Threo*- and *allo*-isoleucyl thiazolidine were titrated against DPP-IV in an in vitro assay as described in RESEARCH DESIGN AND METHODS, and dissociation constants (K_i) were determined. The solid line is theoretical for K_i values for *threo* and *allo* of 201 and 188 nmol/l, respectively, indicating that both compounds bind to the enzyme with virtually identical affinity. *B*: *Threo*- and *allo*-isoleucyl thiazolidine were tested in an oral glucose tolerance test in high-fat diet–induced obese (DIO group) mice (HD, high fat diet; ND, normal diet). Treatment with single oral doses of 3, 10, or 30 mg/kg improved glucose tolerance in a dose-dependent manner. The area under the curve values in the treated groups were significantly less than in the vehicle control, and similar efficacy was observed with both compounds.

activity was observed for both isomers; however, the potency for inhibition of DPP8 and DPP9 differed by ~10-fold, with the *allo* isomer being more potent in each case. Since these differences in inhibition of DPP8/DPP9 were consistent with the observed differences in dose necessary to produce toxicity, it was hypothesized that inhibition of DPP8 and/or DPP9 was responsible for the observed toxicities of both compounds in preclinical species.

Identification of inhibitors selective for DPP-IV, DPP8/9, and QPP. Inhibitors selective for DPP-IV, QPP, and DPP8/9 were identified and their pharmacokinetic properties determined (Fig. 1 and Tables 2 and 3). The DPP-IV–selective compound 3 is a des-fluoro analog of DPP-IV inhibitor sitagliptin currently undergoing clinical investigation for the treatment of type 2 diabetes (28). This compound is a 27-nmol/l inhibitor of DPP-IV, with >1,000-fold selectivity over related proline-specific dipeptidyl peptidases. While potent and selective inhibitors of DPP8 and DPP9 have not been identified, a dual inhibitor, *allo*-isoleucyl isoindoline derivative 4, was discovered. This compound has IC_{50} values of 38 and 55 nmol/l for DPP8 and DPP9, respectively, and IC_{50} values >10 μmol/l over the other related peptidases. QPP-selective inhibitor 5 (QPP IC_{50} = 19 nmol/l) is 100- and 550-fold selective over DPP-IV and DPP9, respectively, and >1,000-fold selective over the remaining proline-specific dipeptidyl peptidases.

All three compounds are orally bioavailable in rats (Table 2), with half-lives ranging from ~0.5 to 2.5 h. Following oral administration at 2 mg/kg, similar exposures were observed for the DPP-IV–selective inhibitor 3 and QPP-selective inhibitor 5. At a similar dose level, the area under the curve observed for the DPP8/9-selective inhibitor 4 was approximately sevenfold higher. Further, the exposures observed for the DPP8/9-selective inhibitor 4 after a 10- and a 30-mg/kg dose were comparable to those observed after a 30- and 100-mg/kg dose of the DPP-IV–selective inhibitor 3. The C_{max} values of the DPP-IV inhibitor 3 and the DPP8/9 inhibitor 4 were similar following oral administration of 10, 30, and 100 mg/kg.

Toxicity studies with DPP-IV, QPP, and DPP-8/9 inhibitors in rats. To obtain evidence that DPP8/9 inhibition was responsible for the toxicities observed with the

TABLE 2
Pharmacokinetic properties of the *threo* and *allo* isomers of isoleucyl thiazolidine in rats and dogs (1 mg/kg i.v. and 2 mg/kg p.o.)

Species	Isomer	Cl_p (ml · min^{-1} · kg^{-1})	Vd_{ss} (l/kg)	$t_{1/2}$ (h)	p.o. AUC (μmol · l^{-1} · h^{-1})	C_{max} (μmol/l)	*F* (%)
Rat	*threo*	27	5.0	1.8	6.17	0.77	102
	allo	28	3.1	1.8	4.29	0.70	71
Dog	*threo*	33	2.2	1.4	4.19	0.44	84
	allo	29	2.4	1.0	2.34	0.40	80

Pharmacokinetic properties of selective compounds in rats

Compound	Dose (mg/kg)	Cl_p (ml · min^{-1} · kg^{-1})	Vd_{ss} (l/kg)	$t_{1/2}$ (h)	p.o. AUC (μmol · l^{-1} · h^{-1})	C_{max} (μmol/l)	*F* (%)
DPP-IV selective (3)	1 i.v., 2 p.o.	43	4.4	1.6	1.03	0.19	51
	10 p.o.				5.76	2.12	
	30 p.o.				26.7	10.7	
	100 p.o.				80.5	27.3	
DPP8/9 selective (4)	1 i.v., 2 p.o.	15	2.6	2.5	6.83	1.50	72
	10 p.o.				26.4	5.68	
	30 p.o.				91.1	15.6	
	100 p.o.				278	36.5	
QPP selective (5)	1 i.v., 2 p.o.	26	0.8	0.5*	1.17	0.78	45

*Mean residence time; $t_{1/2}$ could not be determined due to nonlinear kinetics.

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TABLE 3
Selectivity of compounds for DPP-IV and related dipeptidyl peptidases

Compound	IC_{50} (nmol/l)							
	DPP-IV	DPP8	DPP9	QPP	FAP	PEP	APP	Prolidase
threo-Ile thia (1)	420	2,180	1,600	14,000	>100,000	>100,000	>100,000	>100,000
allo-Ile thia (2)	460	220	320	18,000	>100,000	>100,000	>100,000	>100,000
DPP-IV selective (3)	27	69,000	>100,000	>100,000	>100,000	>100,000	>100,000	>100,000
DPP8/9 selective (4)	30,000	38	55	14,000	>100,000	>100,000	>100,000	>100,000
QPP selective (5)	1,900	22,000	11,000	19	>100,000	63,000	>100,000	>100,000
DPP-IV selective (6)	0.48	>100,000	86,000	>100,000	21,000	39,000	>100,000	>100,000
Val-Boro-Pro (7)	<4	4	11	310	560	390	>100,000	>100,000
Lys[Z(NO_2)] pyrrolidide (8)	1,300	154	165	1,210	51,000	>100,000	>100,000	>100,000
Lys[Z(NO_2)] thiazolidide (9)	410	210	75	210	33,000	56,000	ND	>100,000
Lys[Z(NO_2)] piperidide (10)	17,000	1,100	670	710	>100,000	>100,000	ND	ND

APP, aminopeptidase P; FAP, fibroblast activation protein; ND, not determined; PEP, prolyl endopeptidase.

allo and *threo* isomers, DPP-IV-, QPP-, and DPP8/9-selective compounds were evaluated in 2-week rat toxicity studies and in acute dog tolerability studies. The results from these studies showed a remarkable similarity between the effects produced by the DPP8/DPP9-selective inhibitor and the *allo* compound. As shown in Table 4, both compounds administered to rats produced mortality and a ventral, abdominal alopecia at similar doses. Both compounds also produced thrombocytopenia of similar magnitude at doses ≥30 mg/kg and enlarged spleens and lymph nodes at all doses tested. Although the *allo* compound produced anemia after 2 weeks of treatment that progressed in severity after 4 weeks of treatment, no anemia was observed with the DPP8/9-selective compound after 2 weeks; however, a reduction in reticulocyte counts was found in the 30- and 100-mg/kg dose groups (reticulocyte counts were not measured in the study with the *allo* compound). These data suggest that both compounds have an effect on hematopoiesis in the rat, possibly mediated by a protease other than DPP8/9. Alternatively, the compounds may have differential tissue distributions. Histologically, both compounds produced necrosis of lymphocytes within the spleen and lymph nodes. In addition, histiocytosis in the lungs and inflammatory cell infiltration of multiple organs, including the gastrointestinal tract, were observed.

The QPP-selective inhibitor produced significant reductions in reticulocyte counts at the 100-mg/kg dose. No other antemortem or postmortem changes were noted with this compound. The DPP-IV–selective inhibitor did not produce any changes in physical appearance, body weight, hematology, clinical chemistry, urinalysis, ophthalmology, or postmortem changes in rats. Single-dose toxicokinetic data obtained at doses comparable to those used in the 2-week rat study show that exposures to the DPP-IV–selective compound were similar to, or greater than, the exposures of the *allo* compound associated with the above described toxicity.

Toxicity studies with DPP-IV, QPP, and DPP-8/9 inhibitors in dogs. Acute oral administration of the DPP8/DPP9-selective inhibitor resulted in bloody diarrhea, emesis, and tenesmus, similar to what had been previously observed with the *allo* compound. Conversely, no acute effects were observed in dogs given the DPP-IV–selective inhibitor, despite achieving plasma C_{max} and area under the curve values equal to or greater than those associated with the toxicity with the *allo* compound. No toxicity was observed in dogs given single oral doses of the QPP-selective inhibitor.

Studies with DPP8/9-selective inhibitor in DPP-IV–deficient mice. To demonstrate that the observed toxicities induced by the *allo* and DPP8/9-selective compounds were not due to residual DPP-IV inhibition, the DPP8/9-selective inhibitor was evaluated for toxicity in a 14-day tolerability study in wild-type and DPP-IV–deficient mice. The results are summarized in Table 5. Both 300-mg · kg^{-1} · day^{-1} dose groups were terminated due to excessive mortality and clinical signs of morbidity, indicating that these findings were not due to inhibition of DPP-IV. Findings in DPP-IV–deficient mice at 100 mg · kg^{-1} · day^{-1} included splenomegaly, increased extramedullary hematopoiesis in the spleen, and bone marrow myeloid hyperpla-

TABLE 4
Comparative toxicity studies in rats (2 weeks of treatment at doses of 10, 30, 100 mg · kg^{-1} · day^{-1}) and dogs (single dose, 10 mg/kg p.o.) with selective inhibitors

Species	Toxicity	DPP-IV selective (3) (mg/kg)	DPP8/9 selective (4) (mg/kg)	QPP selective (5) (mg/kg)	*threo*-Ile thia (1) (mg/kg)	*allo*-Ile thia (2) (mg/kg)
Rat	Alopecia	NO	100 (5/10)	NO	NO	100 (2/10)
	Thrombocytopenia	NO	30 (3/10)	NO	675 (10/20)	10 (2/10)
	Anemia	NO	NO	NO	NO	100 (5/20)
	Reticulocytopenia	NO	30 (2/10)	100 (1/5)	NO	ND
	Splenomegaly	NO	10 (4/10)	NO	NO	10 (7/10)
	Mortality	NO	100 (2/10)	NO	NO	300 (7/10)
Dog	Bloody diarrhea	NO	10 (3/3)	NO	225 (1/6)	10 (3/3)

The lowest dose producing the adverse effect is shown with the incidence at this dose in parentheses. Historical data from threo- and allo isoleucyl-thiazolidine safety studies are shown for comparison. NO, not observed at any dose tested; ND, not determined.

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TABLE 5
Comparative toxicity studies with the DPP8/9-selective inhibitor (4) in wild-type and DPP-IV–deficient mice

Toxicity	DPP-IV–deficient mice (mg/kg)	Wild-type mice (mg/kg)
Spleen (EMH)	30 (2/12); 100 (11/12)	ND
Bone marrow myeloid hyperplasia	100 (7/12)	ND
Mortality	100 (1/12); 300 (12/12)	300 (12/12)

Wild-type mice (300 mg · kg^{-1} · day^{-1} p.o.) and DPP-IV–deficient mice (30, 100, and 300 mg · kg^{-1} · day^{-1} p.o.) were treated for 2 weeks. The dose(s) producing the adverse effect is shown with the incidence in parenthesis. EMH, extramedullary hematopoiesis; ND, not determined.

sia; these findings were also observed in rats treated with the DPP8/9 selectivity inhibitor, as described above.

Studies with DPP-IV–, DPP8/9-, and QPP-selective inhibitors in in vitro models of T-cell activation. Lys[Z(NO_2)]–pyrrolidide (compound 8) and related compounds 9 and 10 (Fig. 1) that have been previously used to implicate DPP-IV activity in T-cell activation were found to be nonselective DPP-IV inhibitors, with IC_{50s} <1 μmol/l for DPP8/9 (Table 3), raising the possibility that some of the reported effects of these compounds were due to off-target activity. To test this hypothesis, QPP- and DPP8/9-selective inhibitors (compounds 5 and 4, respectively), a potent nonselective inhibitor, Val-boro-Pro (compound 7), and one of the most potent and selective DPP-IV inhibitors discovered to date (Fig. 1, compound 6) (29), were tested in in vitro models of immune responses. Effects on proliferation and IL-2 release of human T-cells upon both phytohemagglutinin and superantigen stimulation were determined. Val-boro-Pro and the selective DPP8/9 inhibitor inhibited proliferation in these models with IC_{50s} of ~10 and 500 nmol/l, respectively (Fig. 3). Val-boro-Pro also inhibited IL-2 release in these models with an IC_{50} ~500 nmol/l (not shown). In contrast, the QPP (data not shown)- and DPP-IV–selective compounds had no effect in these assays (IC_{50} >50 μmol/l).

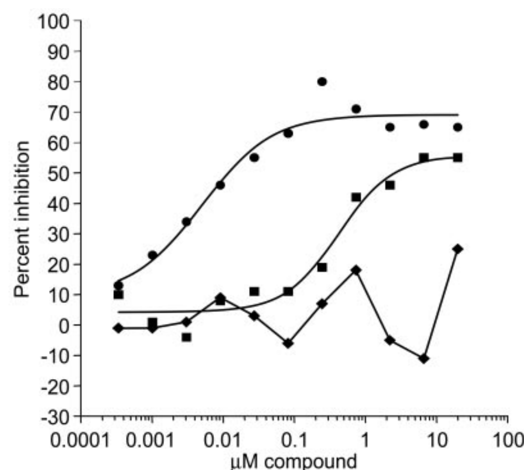

FIG. 3. Effects of selective inhibitors on proliferation of human peripheral blood mononuclear cells. Val-boro-Pro (●), DPP8/9-selective inhibitor 4 (■), and DPP-IV–selective inhibitor 6 (♦) were tested in human peripheral blood mononuclear cells for effects on proliferation. Cells were stimulated with either phytohemagglutinin or superantigen (shown). Val-boro-Pro and the selective DPP8/9 compound inhibited proliferation in this model with IC_{50s} of ~10 and 500 nmol/l, respectively. The DPP-IV inhibitor had no effect in these assays (IC_{50} >50 μmol/l).

DISCUSSION

There is evidence from preclinical studies in animal models of diabetes, studies of DPP-IV–deficient mice, and from clinical studies of DPP-IV inhibitors indicating potential utility of DPP-IV inhibition for the treatment of patients with type 2 diabetes. With the recent identification of several closely related proline-specific enzymes, understanding the degree of selectivity required for the development of inhibitors with an optimal safety profile has become a key issue. The data presented in this manuscript provide compelling evidence that inhibition of DPP8/9, but not selective DPP-IV inhibition, is associated with multiorgan toxicities in preclinical species.

First, at least two structurally distinct compounds that inhibit DPP8/9 show remarkably similar toxicities in rats and dogs. The DPP8/9 inhibitor is highly selective over all other proline specific enzymes, and inhibition of the *allo* compound is limited to DPP-IV, DPP8/9, and weak inhibition of QPP. The finding that the DPP8/9 inhibitors produce similar toxicities in DPP-IV–deficient and wild-type mice establishes that the observed toxicities are not due to inhibition of DPP-IV. Second, the degree of toxicity observed with the *allo* and *threo* compounds correlates with their affinity for DPP8/9. No other distinguishing characteristics for these two isomers that could explain their differential toxicity have been identified. Third, gastrointestinal toxicity in the dog has been reported following oral administration of another DPP-IV inhibitor, CP-867534-01 [(*S*)-2-amino-2-cyclohexyl-1-(3,3,4,4-tetrafluoropyrrolidin-1-yl)ethanone hydrochloride] (30). We have found that this compound also inhibits DPP8 and DPP9 with IC_{50s} <100 nmol/l. Taken together, these findings strongly suggest that DPP8/9 inhibition results in toxicity in preclinical species, although they do not unequivocally rule out the possible contribution of other closely related enzymes. The molecular mechanism that underlies the observed toxicities is unknown.

One theoretical liability of DPP-IV inhibition is compromised immune function because DPP-IV is identical to CD26, a cell surface marker for activated T-cells (19). Indeed, there has been an intense effort to establish whether the catalytic activity of DPP-IV is required for T-cell activation, in part, using inhibitors of DPP-IV, which attenuate T-cell activation in a variety of models of immune function (20). We now report that the compounds used in these experiments have greater intrinsic potency against DPP8 and DPP9 than DPP-IV. Moreover, the DPP8/9-selective inhibitor (compound 4), but not the selective DPP-IV inhibitor (compound 6), attenuated profileration and IL-2 release in human in vitro models of T-cell activation. These results strongly suggest that proteolytic activity is not required for the putative costimulatory function of DPP-IV/CD26 and that immunological effects previously observed with several DPP-IV inhibitor compounds in preclinical models may be due to off-target inhibition of DPP8/9.

These results do not establish whether inhibition of DPP8 or DPP9 (or both) is more important in producing toxicity. Of note, DPP8/9 are cytosolic enzymes, in contrast to DPP-IV, which has an extracellular catalytic domain. Thus, for a given compound, the potential for toxicities at efficacious doses will depend not only upon

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the intrinsic potency against DPP8 and/or DPP9 relative to DPP-IV but also on the intracellular concentration achieved. Indeed, the *threo* and *allo* isomers, and the DPP8/9 inhibitor, are highly cell penetrant. Also unknown is the extent to which the toxicities observed in preclinical species will extend to humans. Given the high degree of homology of DPP8 and DPP9 across species, and the finding that a selective DPP8/9 inhibitor attenuates T-cell activation in a human in vitro system, it is reasonable to speculate that some liabilities of DPP8/9 inhibition may be observed in humans, provided inhibition of these enzymes occurs in vivo.

In summary, these results strongly suggest that inhibition of DPP8/9 produces profound toxicity in preclinical species and is also likely responsible for at least some of the effects on immune function that have been previously attributed to DPP-IV. Some of the toxicities that were noted in dogs did not manifest until 5–6 weeks of treatment, indicating that relatively long-term inhibition of these enzymes may be required to properly assess potential liabilities of DPP8/9 inhibition. Conversely, no toxicities or attenuation of T-cell activation in vitro were observed with selective DPP-IV inhibitors in these studies or in rats and dogs treated for 12 months at doses well above those necessary for pharmacological activity (G.R.L., unpublished data). Taken together, these results strongly suggest that early preclinical assessment of potential clinical candidates for off-target peptidase inhibition is important, with selection and further development of agents that have essentially no DPP8/9 inhibition at pharmacologically relevant plasma exposures.

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