Articles

cis-2,5-Dicyanopyrrolidine Inhibitors of Dipeptidyl Peptidase IV: Synthesis and in Vitro, in Vivo, and X-ray Crystallographic Characterization

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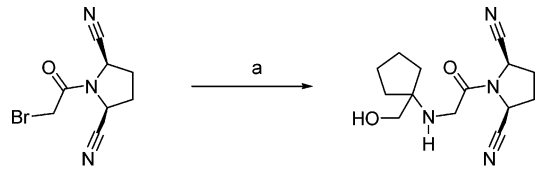
Inhibitors of the glucagon-like peptide-1 (GLP-1) degrading enzyme dipeptidyl peptidase IV (DPP-IV) have been shown to be effective treatments for type 2 diabetes in animal models and in human subjects. A novel series of *cis*-2,5-dicyanopyrrolidine α-amino amides were synthesized and evaluated as inhibitors of dipeptidyl peptidase IV (DPP-IV) for the treatment of type 2 diabetes. 1-({[1-(Hydroxymethyl)cyclopentyl]amino}-acetyl)pyrrolidine-2,5-*cis*-dicarbonitrile (**1c**) is an achiral, slow-binding (time-dependent) inhibitor of DPP-IV that is selective for DPP-IV over other DPP isozymes and proline specific serine proteases, and which has oral bioavailability in preclinical species and in vivo efficacy in animal models. The mode of binding of the *cis*-2,5-dicyanopyrrolidine moiety was determined by X-ray crystallography. The hydrochloride salt of **1c** was further profiled for development as a potential new treatment for type 2 diabetes.

Type 2 diabetes (formerly non-insulin-dependent diabetes mellitus) is a severe and increasingly prevalent disease.[1] Diabetics may suffer debilitating cardiovascular, eye, kidney, and nerve damage and are at risk of premature handicap and death due to these and other diabetic complications, which are the result of glucose toxicity caused by their hyperglycemia. A progressive reduction in insulin sensitivity and insulin secretion are hallmarks of the disease, which eventually result in failure of the pancreatic islet cells and dependence on exogenous insulin. The incretin hormone glucagon-like peptide 1 (GLP-1) is a potent stimulator of endogenous insulin release. GLP-1 has beneficial effects on islet β-cell function and insulin sensitivity without induction of hypoglycemia.[2] Unfortunately, GLP-1 is rapidly degraded in vivo. Inhibition of GLP-1 degradation by dipeptidyl peptidase IV (DPP-IV) has emerged as a promising approach for the treatment of Type 2 diabetes[3] and has been aggressively pursued by numerous laboratories.[4] We sought to identify novel inhibitors of DPP-IV that were structurally distinct from the wide variety of inhibitors previously reported by other groups. In particular, we wanted to explore an achiral *cis*-2,5-dicyanopyrrolidine template, to learn if such compounds were active as inhibitors of DPP-IV. Molecular modeling based on published DPP-IV enzyme−inhibitor crystal structures[5] suggested that the *cis*-2,5-dicyanopyrrolidine template was not a good starting point for compound design. However, it is well-known that binding site residues can move to accommodate a ligand, and 4,5-methano-bridged inhibitors of DPP-IV have been reported.[6] Therefore, we opted to test the idea that a *cis*-2,5-dicyanopyrrolidine template might inhibit DPP-IV. This paper reports the results of our investigation.

Synthesis. The compounds were prepared by parallel synthesis according to the procedure outlined in Scheme 1, in which the preparation of **1c** is illustrated for example. Coupling of the bromoacetamide **2** with an excess (≥3 equiv) of the appropriate primary amine was carried out in acetonitrile at 20 °C. On the basis of previously reported work detailing the stability of nitrile-containing inhibitors of DPP-IV, we elected to employ only amines bearing a quaternary α-carbon center.[7] The products were converted to their hydrochloride salts by treatment with 1 equiv of hydrogen chloride in methanol following purification by silica gel chromatography.

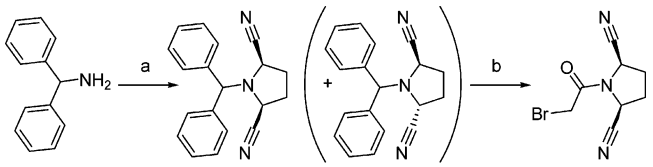
Scheme 1. Preparation of *cis*-2,5-Dicyanopyrrolidine Inhibitors[a]



2 **1c**

[a] Conditions: (a) 1-(Hydroxymethyl)cyclopentylamine (3 equiv), MeCN, 20 °C, 18 h.

Scheme 2. Preparation of Bromoacetamide **2**[a]



3 **4** **2**

[a] Conditions: (a) 2,5-Dimethoxytetrahydrofuran, KCN, 0.1 M aqueous citric acid, 20 °C, 72 h; (b) $BrCH_2COBr$, MeCN, 70 °C, 16 h.

The requisite bromoacetamide **2** was prepared by the route shown in Scheme 2. Strecker reaction of 2,5-dimethoxytetrahydrofuran and aminodiphenylmethane with KCN in 0.1 M aqueous citric acid solution afforded a precipitate containing

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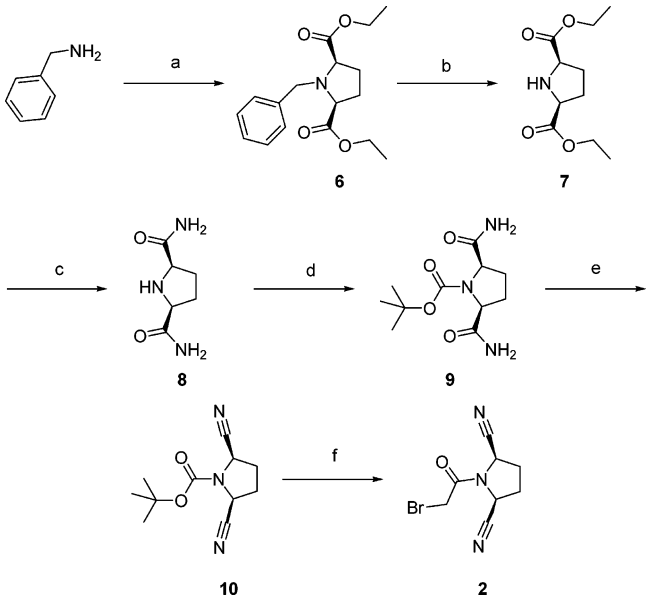
Scheme 3. Alternative Preparation of Bromoacetamide **2**[a]



[a] Conditions: (a) Diethyl *meso*-dibromoadipate, toluene, 80 °C, 16 h; (b) H_2 (50 psi), Pd/C, MeOH, 20 °C, 18 h; (c) NH_3, MeOH, 20 °C, 7 d; (d) BOC_2O, H_2O, 1,4-dioxane, 20 °C, 18 h; (e) $POCl_3$, imidazole, C_5H_5N, CH_2Cl_2, 0 °C → 20 °C, 90 m; (f) $BrCH_2COBr$, MeCN, 20 °C, 1 h.

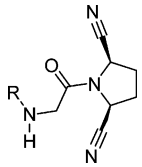
the desired *cis*-dinitrile **3**, along with *N*-benzhydrylpyrrole (**5**) and lesser amounts of the corresponding *trans*-dinitrile isomer (**4**).[8] Purification of the desired *cis*-dinitrile **3** was accomplished by recrystallization from acetonitrile. Direct conversion of **3** to the bromoacetamide **2** was accomplished by reaction of **3** with bromoacetyl bromide in acetonitrile at 70 °C, followed by chromatographic purification to remove the byproduct benzhydryl bromide.[9]

Alternatively, bromoacetamide **2** could be prepared from diethyl *meso*-dibromoadipate[10] as shown in Scheme 3.[11] Reaction with excess benzylamine afforded predominantly the *cis*-isomer of the diethyl ester **6**. Crystallization of the hydrochloride salt afforded pure **6**, which was then converted to the free base and subjected to hydrogenolysis to afford **7**. Treatment with excess ammonia afforded the crystalline diamide **8**, which was converted to the N-BOC derivative **9**. Dehydration with phosphoryl chloride gave the BOC-protected dinitrile **10**, which upon exposure to bromoacetyl bromide gave the bromoacetamide **2**.

The amines that were coupled with bromoacetamide **2** were, in general, commercially available amines (Table 1). In certain cases, the desired amine fragment was not commercially available (**1l**, **1z**). These amines were prepared from the corresponding tertiary alcohols via the intermediate azide by treatment with azidotrimethylsilane and boron trifluoride etherate[12] followed by hydrogenation over palladium on carbon. 1-(Alkoxymethyl)-1-cyclopentylamines (Table 2) were prepared from commercially available 1-(hydroxymethyl)-1-cyclopentylamine by a three-step sequence (Scheme 4).

Evaluation of Enzyme Inhibition. Recombinant human DPP-IV activity was measured by the release of 4-methoxy-2-aminonaphthylamine from the artificial substrate Gly-Pro-4-methoxy-β-naphthylamide.[13] We were gratified to find that the *cis*-dinitrile pyrrolidine scaffold did indeed afford compounds with DPP-IV inhibitory activity. Screening a variety of primary amines bearing a tertiary carbon center at the α position revealed that the best potency was generally attained with those amines in which the α-carbon was part of a cyclic or polycyclic framework (Table 1). In particular, the adamantane (**1b**, **1d**), noradamantane (**1a**), norbornane (**1g**), and cyclopentane (**1c**) fragments afforded compounds with acceptable enzyme inhibitory potency. Acyclic hydrocarbon fragments were generally less potent (for example, **1s**, **1t**, **1u**). Also noted was an apparent preference for a cyclopentane ring as opposed to a cyclohexane ring (**1c** vs **1k**).

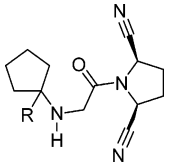
Table 1. Effect of Amine Substituent on DPP-IV Enzyme Inhibitory Activity



entry	R	DPP−IV IC_{50}, nM[a]
1a	3-noradamantyl	72
1b	1-(3-HO)adamantyl[b]	74
1c	1-($HOCH_2$)cyclopentyl	104
1d	1-adamantyl	106
1e	1-(C_6H_5)−1-cyclobutyl	164
1f	t-$BuCH_2(CH_3)_2C$	208
1g	2-*exo*-norbornyl	237
1h	$C_6H_5(CH_3)_2C$	261
1i	(4-FC_6H_4)$CH_2(CH_3)_2C$	651
1j	*tert*-butyl	663
1k	1-($HOCH_2$)cyclohexyl	687
1l	1-methylcyclohexyl[c]	689
1m	$HOCH_2(CH_3)_2C$	1110
1n	1-($C_6H_5CH_2$)cyclobutyl	1160
1o	1-($HOCH_2$)-4-THP[d]	1220
1p	1-(ethynyl)cyclohexyl	>3000
1q	1-(ethyl)cyclohexyl[e]	>3000
1r	3,5,7-trifluoro-1-adamantyl[f]	>3000
1s	$(CH_3)_2CH(CH_3)_2C$[c]	> 3000
1t	$CH_3(C_2H_5)_2C$[c]	> 3000
1u	$CH_3CH_2(CH_3)_2C$[c]	> 3000

[a] Recombinant wild-type human enzyme. Values are means of at least three experiments; standard deviations are ±15%. An IC_{50} of >3000 indicates that no curve was noted in the dose−response up to 3 μM. [b] See ref 14. [c] Prepared from the corresponding tertiary alcohol with $TMSN_3$ and BF_3 etherate; see Experimental Section. [d] 4-Tetrahydropyranyl. [e] Prepared by hydrogenation of 1-ethynylcyclohexylamine. [f] See ref 15.

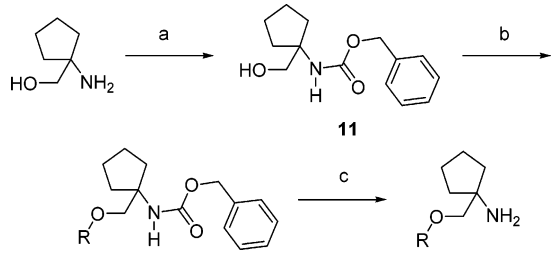
Table 2. Effect of Amine Substituent on DPP-IV Enzyme Inhibitory Activity



entry	R	DPP-IV IC_{50}, nM[a]
1c	$HOCH_2$	104
1v	$CH_3CH_2OCH_2$[b]	64
1w	CH_3OCH_2[b]	84
1x	$CH_3CH_2CH_2CH_2OCH_2$[b]	91
1y	$CH_3CH_2CH_2OCH_2$[b]	123
1z	CH_3[c]	201
1aa	$(CH_3)_2CHCH_2OCH_2$[b]	454
1bb	$C_6H_{11}OCH_2$[d,b]	702
1cc	$CH_3OCH_2CH_2$[b]	955
1dd	$(CH_3)_3COCH_2$[b]	1860
1ee	1-$CONHCH_3$	>3000
1ff	1-CO_2CH_3	>3000
1gg	$HOCH_2CH_2$[b]	>3000

[a] Recombinant wild-type human enzyme. Values are means of at least three experiments; standard deviations are ±15%. An IC_{50} of >3000 indicates that no curve was noted in the dose response up to 3 μM. [b] Prepared from N−Cbz1-(hydroxymethyl)-1-cyclopentylamine; see Experimental Section. [c] Prepared from the corresponding tertiary alcohol with $TMSN_3$ and BF_3 etherate; see Experimental Section. [d] (Cyclcohexyloxy)-methyl.

Scheme 4. Preparation of 1-(Alkoxymethyl)-1-cyclopentylamines[a]



[a] Conditions: (a) Cbz-Cl, CH_2Cl_2, 0 °C, 1 h; (b) R-Br, Ag_2O, DMF, 20 °C, 18 h; (c) H_2, Pd−C, MeOH, 20 °C, 18 h.

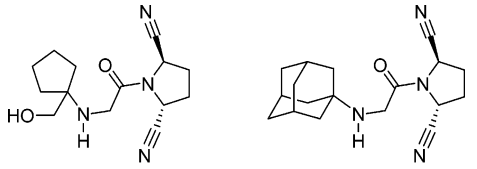


Figure 1. Inactive trans analogues.

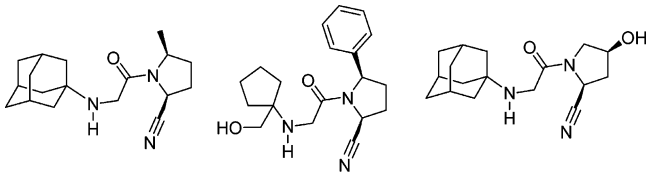


Figure 2. Inactive cis analogues.

Based on the synthetic flexibility of the cyclopentane framework, and upon the results of preliminary in vivo studies with **1b** and **1c** (vide infra), further analogue generation centered upon targeted analogues incorporating a cyclopentyl fragment in the amine residue (Table 2).

Compounds with a substituent containing the CH_2O subunit (**1v**, **1w**, **1x**) were clearly preferred over other functional groups (for example, **1ee** and **1ff**). Steric encumbrance of this subunit decreased potency (**1aa**, **1dd**), suggesting that perhaps this subunit was also involved in a binding interaction. The remarkable loss of potency observed when the CH_2O subunit was extended to a CH_2CH_2O subunit as in **1cc** and **1gg** further supported this conclusion. In addition, the potency preference for a cyclopentane ring over a cyclohexane ring was again observed (for example, **1z** vs **1l**).

The *trans*-dinitrile isomers of **1c** and **1d** (**14a**, **14b**) were prepared from **4** in the same manner as **1c** and **1d** and found to have DPP-IV $IC_{50} \gg 30$ μM (Figure 1). The *cis*-5-methyl analogue **15**,[16a] the *cis*-5-phenyl analogue **16**,[16b] and the *cis*-4-hydroxy analogue **17**[16c] were also inactive ($IC_{50} \gg 30$ μM, Figure 2). The kinetics of DPP-IV inhibition by **1c** were studied at various concentrations and revealed that the binding of **1c** to DPP-IV was time dependent (Figure 3) and reversible (Figure 4). No evidence was found to indicate that the covalently bound imidate (vide infra) underwent hydrolysis to the corresponding carboxylic acid. Activity was assayed by spectrophotometric measurement (405 nm) of the liberation of 4-nitroaniline from H-Ala-Pro-4-nitroanilide.[17] Time dependent binding of **1c** to DPP-IV was assessed by the addition of substrate (100 μM) and inhibitor (at the concentrations indicated) to the enzyme at $t = 0$. Dissociation of the DPP-IV−**1c** complex was assayed

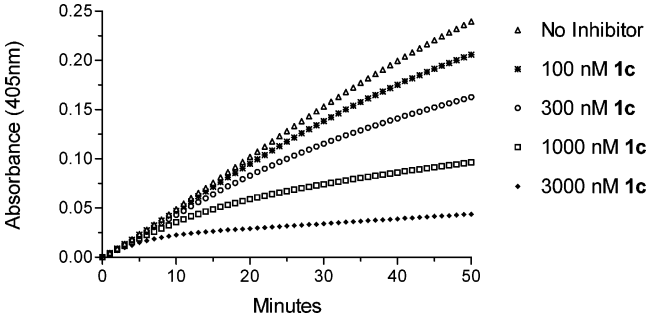


Figure 3. Slow binding kinetics of DPP-IV enzyme inhibition by **1c**.

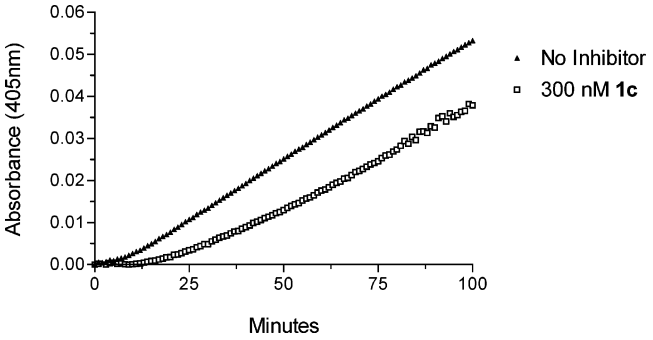


Figure 4. Slow binding kinetics of DPP-IV enzyme inhibition by **1c**.

by incubation of enzyme for 100 min in the presence or absence of 300 nM **1c**, followed by 100-fold dilution into a reaction mixture containing 1 mM H-Ala-Pro-4-nitroanilide.

Compounds exhibiting DPP-IV $IC_{50} \leq 120$ nM were subsequently screened for their ability to inhibit other enzymes with DPP-IV-like activity: DPP-II, DPP-III, DPP-VIII, DPP-IX, FAP, and APP.[18] No compounds had any significant inhibitory activity against any of these enzymes (all IC_{50} values were $\gg$ 30 μM).

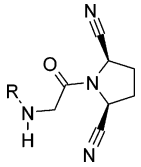
In Vivo Hypoglycemic Evaluation. Compounds exhibiting DPP-IV $IC_{50} \leq 100$ nM were subsequently dosed orally at an initial dose of 10 mg kg^{-1} in fasted, diabetic KK/H1J mice, and the response of the mice to a subsequent oral glucose challenge was measured. [1-(*S*)-1-cyclohexyl-2-oxo-(3,3,4,4,-tetrafluoropyrrolidin-1-yl)ethyl]amine (**18**, DPP-IV $IC_{50} = 110$ nM) was used as a positive control in these experiments.[19]

This was performed to demonstrate that the compounds could effect glucose lowering in vivo by DPP-IV inhibition, as subsequent pharmacokinetic evaluation of the compounds did not examine in vivo hypoglycemic activity per se. The initial dose of 10 mg kg^{-1} was selected in an attempt to compensate for the unknown pharmacokinetics of the test compound in the KK/H1J mice. Selected compounds were subsequently tested at lower doses. Due to interassay variability, the compounds were scored simply as exhibiting significant or nonsignificant inhibition of glucose excursion in the oral glucose tolerance test (Table 3).

Pharmacokinetic Evaluation. Certain compounds that were active in the KK/H1J mouse oral glucose tolerance test were advanced to study of their pharmacokinetic properties in the Sprague−Dawley rat. Oral pharmacokinetic evaluations were conducted using the compound delivered in 0.5% methylcellulose formulation at 5 mg kg^{-1} (Table 4). Intravenous pharmacokinetic evaluations were conducted using the compound in sterile saline solutions at 1 mg kg^{-1}.

On the basis of its pharmacokinetic properties in the rat, compound **1c** was advanced to further pharmacokinetic profiling in the beagle dog and cynomolgus monkey (Table 5). For these

Table 3. In Vivo Hypoglycemic Activity of DPP-IV Inhibitors



entry	R	DPP-IV IC_{50}, nM[a]	in vivo glucose lowering	% lowered[b]
1v	$CH_3CH_2OCH_2$	64	significant	79
1a	3-noradamantyl	72	significant[c]	62
1b	1-(3-HO)adamantyl	74	significant[d]	45
1w	CH_3OCH_2	84	significant	74
1x	$CH_3CH_2CH_2CH_2OCH_2$	91	not significant	16
1c	1-($HOCH_2$)cyclopentyl	104	significant	67
1c	1-($HOCH_2$)cyclopentyl	104	significant[e]	59
1c	1-($HOCH_2$)cyclopentyl	104	significant[f]	46
1d	1-adamantyl	106	significant[d]	55
18		110	significant	57

[a] Recombinant wild-type human enzyme. Values are means of at least three experiments; standard deviations are ±15%. [b] Data are means of 10 animals per group; standard deviations are ≤15%. See Experimental Sectionfor details. [c] Inhibitor dosed at 5 mg kg^{-1}. [d] Inhibitor dosed at 3 mg kg^{-1}. [e] Inhibitor dosed at 2.5 mg kg^{-1}. [f] Inhibitor dosed at 0.3 mg kg^{-1}.

Table 4. In Vivo Pharmacokinetic Parameters of DPP-IV Inhibitors in the Rat

compd	DPP-IV IC_{50}, nM[a]	CL_h, mL/min/kg	V_{ss}, L/kg	$t_{1/2}$, h (iv)	$t_{1/2}$, h (po)	*F*, %
1b	74	130	2.7	0.4	1.4	42
1w	84	98	1.6	0.2	0.64	48
1c	104	70	1.4	0.3	2.7	77
1d	106	80	3.1	0.6	nd[b]	nd

[a] Recombinant wild-type human enzyme. Values are means of at least three experiments; standard deviations are ±15%. [b] Not determined.

Table 5. In Vivo Pharmacokinetic Parameters of **1c**

species	DPP-IV IC_{50}, nM[a]	unbound fraction	CL_h, mL/min/kg	V_{ss}, L/kg	$t_{1/2}$, h (iv)	$t_{1/2}$, h (po)	*F*, %
rat	38	100	70	1.4	0.3	2.7	77
dog	109	100	22	1.3	0.8	5.3	93
monkey	92	84	42	1.7	0.6	2.4	42
human	69	77	15[b]	1.2[b]		2.0[c]	70[b]

[a] Plasma enzyme assay. Values are means of at least three experiments; standard deviations are ±20%. [b] Projected values based on allometric scaling. [c] Projected values based on combination of clearance and volume predictions.

experiments, oral pharmacokinetic evaluations were conducted using the compound delivered in sterile water at 1 mg kg^{-1}. Intravenous pharmacokinetic evaluations were conducted at 1 mg kg^{-1} using sterile saline as the vehicle. Simultaneously, **1c** was evaluated for its ability to inhibit rat, dog, monkey, and human DPP-IV using enzyme derived from plasma. Similar potency against all four isoforms was observed. Since in vitro metabolism of **1c** was not detected, human clearance was predicted using allometric scaling of rat, dog, and monkey clearance data with and without protein binding consideration.

The compound exhibited good bioavailability in all species, a low volume of distribution, and moderate clearance. Rapid absorption was also noted in all species as indicated by T_{max} times that were 1 h or less. To assess whether nonlinear exposure might be observed with **1c**, this compound was dosed in rats at higher amounts. We were pleased to note dose proportional increases in exposure (C_{max} and $AUC_{0-\infty}$) following oral doses of 5, 15, and 50 mg kg^{-1} in a 0.5% methylcellulose vehicle.

Effects on Endogenous GLP-1 Levels. Having established that **1c** resulted in improved glucose tolerance in vivo following an oral glucose challenge, we sought to demonstrate that

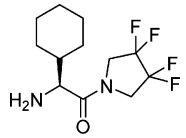


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Figure 5. [1-(*S*)-1-Cyclohexyl-2-oxo-(3,3,4,4,-tetrafluoropyrrolidin-1-yl)ethyl]amine.

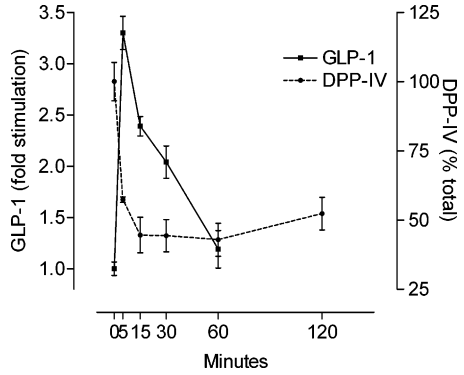

Figure 6. Plasma DPP-IV ativity and GLP-1 levels in KK mice dosed with **1c**.

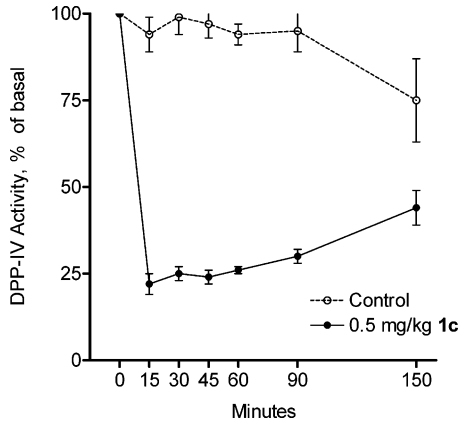

Figure 7. Plasma DPP-IV activity in beagles dosed with **1c**.

administration of **1c** resulted in increased plasma levels of GLP-1. An initial experiment was conducted in fed KK mice, which were dosed with 5 mg kg^{-1} **1c** po at $t = 0$ min. Blood samples were collected at intervals over 2 h for the measurement of GLP-1 and DPP-IV activity (Figure 6). Following dosing of **1c**, total plasma DPP-IV activity was reduced by approximately 50%. Plasma GLP-1 increased to levels approximately three times those recorded at $t = 0$ min.

A second experiment was conducted in beagle dogs, using a procedure adapted from one previously reported for demonstration of the effects of DPP-IV inhibition on plasma GLP-1 levels.[20] The dogs were fasted for 18 h, after which the $t = 0$ blood sample was taken and they were dosed with water or 0.5 mg kg^{-1} **1c** in water. At $t = 30$ min, a meal comprising 9 mL kg^{-1} of a 1:1 v/v mixture of dairy sour cream and 0.5 M sucrose was administered.[21] Additional blood samples were taken for analysis at the times indicated (Figures 7 and 8). As anticipated, plasma DPP-IV enzyme activity was reduced in the treatment group to less than 50% of plasma DPP-IV activity in the vehicle control group. Likewise, plasma GLP-1 levels were increased by two to three times in the treatment group relative to the vehicle control group. Analysis of the area under the curve for the GLP-1 response was analyzed and indicated a significant elevation of GLP-1 in the group treated with **1c** (Figure 9).

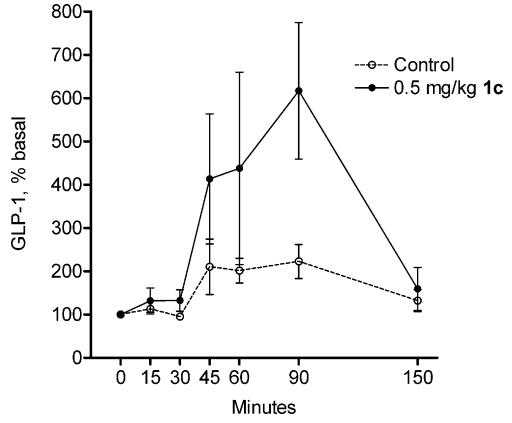

Figure 8. Plasma GLP-1 levels in beagles dosed with **1c**.

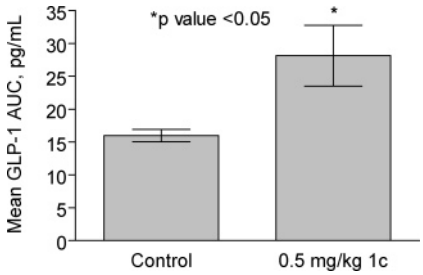

Figure 9. GLP-1 AUC in beagles dosed with **1c**.

Further Profiling. On the basis of the results obtained with **1c**, this compound was further profiled to determine its suitability for clinical development. The mono-hydrochloride salt of **1c** was found to be an anhydrous crystal form that melted at ~173 °C that was nonhygroscopic under kinetic measurements. In the biological model system of phosphate buffered saline (PBS, pH = 6.5) the solubility of the mono-hydrochloride salt form was determined to be >76 mg mL^{-1}. The solubility of the mono-hydrochloride salt form in unbuffered water was difficult to determine precisely, but was in the range of 200 to 300 mg mL^{-1}, with final solution pH = 2.0.

Experiments were also conducted in order to address the potential concern that metabolism of the dicyanopyrrolidine ring moiety in **1c** could liberate cyanide ion by a reverse Strecker reaction. First, metabolite identification studies were completed for both in vitro (microsomes and hepatocytes; rat, dog, monkey, human) and in vivo (plasma and urine; rat, dog, monkey) samples. Products corresponding to the loss of the cyano group were not detected in any of the samples. Next, to further confirm this finding, in vivo plasma samples (rat, dog, monkey) were analyzed for thiocyanate ion, as cyanide ion is detoxified in vivo by conversion to thiocyanate.[22] Thiocyanate is found in plasma at basal levels ranging from 10 to 100 μM. The basal level of thiocyanate ion in human plasma has been reported to be around 30 μM.[23] Previously collected plasma samples were therefore analyzed for thiocyanate ion. In rats (15 mg kg^{-1}, po), dogs (3 mg kg^{-1} po), and monkeys (3 mg kg^{-1} po), no increases in thiocyanate were detected through 24 h when compared to the $T = 0$ sample, providing further support for the conclusion cyanide ion was not liberated from **1c** following in vivo administration. Further confidence in the projected chronic safety of **1c** was gained by a preclinical 5-day toxicity study in rats. Male and female Sprague − Dawley rats (3 of each sex) were dosed with 500 mg/kg of **1c** for 5 days. Clinical signs, clinical chemistry, hematology, and postmortem findings (macroscopic and microscopic) were all unremarkable. A single-dose safety study in the dog in which **1c** was administerd at 20 mg kg^{-1} iv likewise produced no remarkable findings.[24]

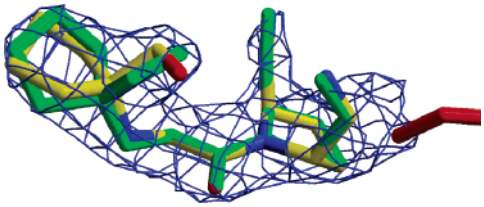

Figure 10. Electron density map of **1c** bound at rHDPP-IV active site at 1.6 σ. Yellow is the binding mode of **1c**; alternative binding mode in green would also fit the electron density, but makes less sense in light of in vitro SAR findings. The CH_2OH group of S630 is shown in red.

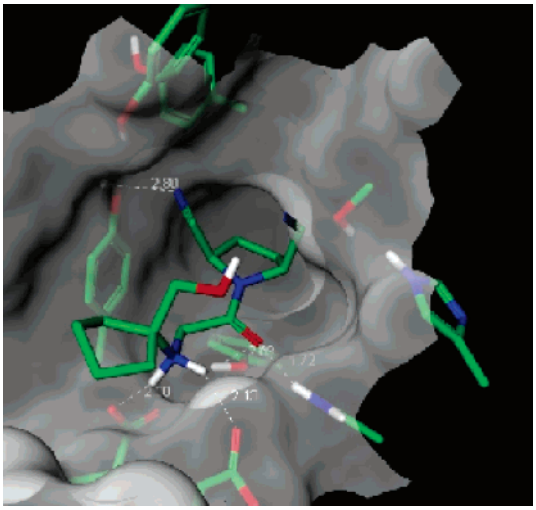

Figure 11. X-ray crystal structure of **1c** bound to rHDPP-IV showing (clockwise from top) side chains of Y547, Y631, E205, E206, N710, H740, S630, and Y666. R125, ordered water molecules, and the hydrogen bond network present between the ordered water molecules, compound **1c**, and protein atoms have been omitted for clarity.

X-ray Crystallography. To better understand the binding mode of our compounds, we cocrystallized recombinant human DPP-IV with **1c** (Figures 10 and 11). The DPP-IV active site is defined by a catalytic triad (S630, H740, D708), an oxyanion hole (Y547 OH, Y631 N), and specificity residues (E205, E206, Y662, Y666, N710). One nitrile group is attacked by S630 to form a covalent link in the crystal structure. The other nitrile fits into the active site by forcing the Y547 side chain to move by ~2.8 Å. This nitrile nitrogen is likely hydrogen bonded to the Y666 OH (3.1 Å) and is 3.5 Å from Y547 OH. The π cloud of the triple bond stacks with the edge of Y666. The pyrrolidine ring occupies the S1 pocket (Y662, Y666, V656, V711, Y631, W659) and compares well with that in previous structures (shifted 0.4 Å, coordinates error 0.28 Å). The P2 backbone is recognized by E205 and E206 (3.0 and 2.9 Å to the secondary amine, respectively), N710 (2.7 Å to carbonyl), and Y662 (2.9 Å to carbonyl). The cyclopentane ring is in the large S2 site, but surprisingly does not appear make any hydrophobic interactions with the protein (all protein atoms < 4.0 Å to the cyclopentane ring are oxygen). The alcohol makes a hydrogen bond with the R125 side chain directly (3.1 Å) and via H_2-O128 (OH to H_2O 2.6 Å, H_2O to R125 2.7 Å). This hydrogen bonding would appear to be the basis for the preference observed in vitro for the CH_2O subunit (**1c**, **1v**, **1w**, **1x**) over the CH_2-CH_2O subunit (**1cc** and **1gg**) or other functional groups (**1ee, 1ff**). This hydrogen bond would also be consistent with the

previous observation that steric encumbrance of this subunit resulted in decreased potency (**1aa**, **1dd**).

Comparison of this crystal structure with published data shows that with one nitrile group on the pyrrolidine ring, the covalent modification of S630 would position the intermediate imidate nitrogen perfectly in the oxyanion hole, forming hydrogen bonds with Y547 OH and Y631 N. Binding of the *cis*-dinitrile **1c** likewise leads to imidate ester formation; however, the interaction of the imidate nitrogen with the oxyanion hole is no longer possible. Movement of Y547, however, results in favorable binding of the remaining nitrile group with Y666 via π-stacking and hydrogen bonds.

Further structural work was conducted by NMR using **1c** bearing ^{13}C labels (>98%) on both nitrile carbons.[25] The ^{13}C NMR of $(^{13}CN)_2$ **1c** in 25 mM Tris buffer at pH 7.5 showed two signals at approximately 118 and 119 ppm. Upon addition of recombinant human DPP-IV to the sample, the resonance at 118 ppm was replaced by a resonance at approximately 177 ppm, indicating that binding to the enzyme was accompanied by imidate formation.

Summary

1-({[1-(Hydroxymethyl)cyclopentyl]amino}acetyl)pyrrolidine-2,5-*cis*-dicarbonitrile (**1c**) is an achiral inhibitor of DPP-IV that selectively inhibits DPP-IV over DPP-II, DPP-III, DPP-VIII, DPP-IX, APP, and FAP. It exhibited oral bioavailability in the rat, dog, and monkey and displayed in vivo efficacy in a mouse OGTT model. It has a high free fraction, low volume of distribution, moderate clearance, and moderate half-life. The mode of binding of the *cis*-2,5-dicyanopyrrolidine moiety was determined by X-ray crystallography and determined to involve a covalent interaction of S630 with one nitrile group, while the other nitrile forces the Y547 side chain to move and subsequently makes a π-stacking interaction and H-bond with Y666. The secondary amine is recognized by E205, E206, N710, and Y662. The covalent nature of the interaction with S630 was indicated by ^{13}C NMR. The hydrochloride salt of **1c** was subjected to further profiling for development as a potential new treatment for type 2 diabetes. Preliminary safety assessments with **1c** indicated no safety liability with this new class of compounds.

Experimental Section

All procedures involving animals were reviewed and approved by the Pfizer Institutional Animal Care and Use Committee.

1-Benzhydryl-pyrrolidine-2,5-*cis*-dicarbonitrile (3). To 900 mL of 0.1 M aqueous citric acid solution was added 8.24 g (45.0 mmol) of aminodiphenylmethane, followed by 4.00 g (61.4 mmol) of KCN. The mixture was stirred at 20 °C until the reactants had dissolved, after which 11.00 g (83.2 mmol) of 2,5-dimethoxytetrahydrofuran was added in one portion. The mixture was stirred at 20 °C for about 72 h, after which 14.9 g of precipitate was collected by filtration, washed with water, and dried. The precipitate was dissolved in a minimum of acetonitrile with heating and allowed to cool, whereupon 3.90 g (30%) of **3** precipitated and was collected by filtration. ^1H NMR ($CDCl_3$): 7.53 (m, 4 H); 7.30 (m, 4 H); 7.24 (m, 2 H); 5.11 (s, 1 H); 3.98 (m, 2 H); 2.35 (m, 4 H). ^{13}C NMR ($CDCl_3$): 140.4, 129.4, 128.4, 127.9, 117.0, 69.1, 50.9, 28.9. APCI MS: 167 (Ph_2CH^+ fragment). mp: 194−197 °C. Anal. ($C_{19}H_{17}N_3$) C, H, N.

Evaporation of the mother liquor gave a residue that was flash chromatographed on silica gel eluting with 4:1 hexanes−EtOAc to provide 0.80 g (6%) of 1-benzhydryl-pyrrolidine-2,5-*trans*-dicarbonitrile **4**: ^1H NMR ($CDCl_3$): 7.53 (m, 4 H); 7.34 (m, 4 H); 7.25 (m, 2 H); 4.89 (s, 1 H); 3.86 (m, 2 H); 2.48 (m, 2 H); 2.28 (m, 2 H). APCI MS: 167 (Ph_2CH^+ fragment). mp: 187−188 °C.

1-Bromoacetyl-pyrrolidine-2,5-*cis*-dicarbonitrile (2 from 3). To a solution of 3.00 g (10.4 mmol) of **3** in 40 mL of MeCN was added 1.1 mL (12 mmol) of bromoacetyl bromide with stirring at 70 °C. The mixture was stirred at 70 °C for 16 h. The mixture was concentrated, and the residue was flash chromatographed on silica gel eluting with 1:1 hexanes−EtOAc to afford 2.47 g (99%) of **2** as an oil that crystallized upon storage below 0 °C. ^1H NMR ($CDCl_3$): 4.94 (m, 1 H); 4.68 (m, 1 H); 4.01 (d, J = 13.2 Hz, 1 H); 3.96 (d, J = 13.2 Hz, 1 H); 2.52 (m, 1 H); 2.40 (m, 3 H). ^{13}C NMR ($CDCl_3$): 165.1, 116.8, 47. 6, 47.5, 31.6, 28.9, 25.9. APCI MS: 242, 244 (MH^+, Br isotope pattern). mp 49−50 °C. Anal. ($C_8H_8BrN_3O$) C, H, N.

1-Benzylpyrrolidine-2,5-*cis*-dicarboxylic Acid Diethyl Ester Hydrochloride (6). A solution of 253 g (700 mmol) of diethyl *meso*-2,5-dibromoadipate in 1000 mL of toluene was stirred at 80 °C while 253 mL (2300 mmol) of benzylamine was added over the course of 1 h. Upon completion of the addition of benzylamine, the solution was kept at 80 °C with stirring overnight. The mixture was cooled to room temperature and the precipitate was filtered and washed twice with toluene. The washings were combined with the filtrate and washed water, then twice with pH = 7 5% potassium phosphate solution, brine, and dried ($MgSO_4$). Filtration and evaporation afforded an amber oil that was dissolved in 900 mL of ethyl acetate and treated with 150 mL of 4 M HCl in 1,4-dioxane at 20 °C. The mixture was cooled with stirring in ice for 20 m. The resulting white precipitate was filtered, washed with ethyl acetate, and dried under dynamic vacuum to afford 164 g (70%) of the hydrochloride salt of **6**. ^1H NMR (D_2O): 7.34 (m, 5 H); 4.50 (s, 2 H); 4.46 (m, 2 H); 3.99 (q, 4 H); 2.43 (m, 2 H); 2.09 (m, 2 H); 1.04 (t, 6 H). ^{13}C NMR (D_2O): 168.9, 131.2, 131.0, 130.8, 129.7, 67.9, 64.7, 60.2, 27.5, 13.3. APCI MS: 306 (MH^+). mp 120−122 °C. Anal. ($C_{17}H_{23}NO_4$ HCl H_2O) C, H, N.

Pyrrolidine-2,5-*cis*-dicarboxylic Acid Diethyl Ester (7). A solution of 164 g (480 mmol) of **6** in 250 mL of CH_2Cl_2 was stirred vigorously with 250 mL of 5 M NH_4OH for 15 m at 20 °C. The CH_2Cl_2 solution was separated, washed with brine, dried (Na_2SO_4), and concentrated to give a colorless oil. The oil was dissolved in 500 mL of MeOH, 4.8 g of 10% Pd/C was added, and the mixture was shaken under 50 psi of H_2 at room-temperature overnight. The mixture was filtered through Celite, and the solvent was evaporated to provide 98 g (95%) of **7** as an oil. ^1H NMR (D_2O): 4.44 (m, 2H); 4.13 (q, 4H); 2.31 (m, 2H); 2.09 (m, 2H); 1.12 (t, 6H). ^{13}C NMR (D_2O): 169.3, 64.3, 60.4, 27.4, 13.3. APCI MS: 216 (MH^+). Anal. ($C_{10}H_{17}NO_4$ HCl) C, H, N.

Pyrrolidine-2,5-*cis*-dicarboxylic Acid Diamide (8). **7** (96.4 g, 448 mmol) was dissolved in 1300 mL of 7 M NH_3 in MeOH. The reaction vessel was closed tightly, and the mixture was allowed to stand at 20 °C for about 7 days until the reaction was complete. The crystals that formed were filtered, washed with ethanol, and dried to afford 65.4 g (93%) of **8** as a white solid. ^1H NMR (D_2O): 3.69 (m, 2H); 2.03 (m, 2H); 1.67 (m, 2H). ^{13}C NMR (D_2O): 181.3, 60.8, 30.2. APCI MS: 158 (MH^+). mp: 229−230 °C. Anal. ($C_6H_{11}N_3O_2$) C, H, N.

2,5-*cis*-dicarbamoylpyrrolidine-1-carboxylic Acid *tert*-Butyl Ester (9). A solution of 3.73 g (23.7 mmol) of **8** in 30 mL of H_2O was treated with a solution of 5.70 g (26.1 mmol) of di-*tert*-butyl dicarbonate in 60 mL of 1,4-dioxane. The mixture was stirred at 20 °C overnight, after which the mixture was concentrated to provide 5.98 g (100%) of **9** as a white solid. ^1H NMR (CD_3OD): 4.26 (m, 2 H); 2.30 (m, 2 H); 1.99 (m, 2 H); 1.42 (s, 9 H). ^{13}C NMR (CD_3OD): 182.0, 181.8, 157.9, 85.1, 65.5, 65.2, 34.0, 33.6, 31.3. APCI MS: 258 (MH^+). mp: 196−197 °C. Anal. ($C_{11}H_{19}N_3O_4$) C, H, N.

2,5-*cis*-Dicyanopyrrolidine-1-carboxylic Acid *tert*-butyl Ester (10). Imidazole (7.08 g, 104 mmol) and **9** (6.70 g, 26.0 mmol) were dissolved in 65 mL of dry pyridine and 260 mL of CH_2Cl_2.. The solution was cooled to 0 °C, and 19 mL (200 mmol) of $POCl_3$ was added dropwise. The mixture was stirred at 0 °C for 30 m and at 20 °C for 1 h. Water (4 mL) in pyridine (12 mL) was added dropwise with stirring and ice cooling to quench excess $POCl_3$. The mixture was then evaporated, and the residue was triturated

with dry THF and filtered. The filtrate was stirred for 10 min with 50 g of Amberlite IR-120 H^+ ion-exchange resin and filtered through a pad of anhydrous $MgSO_4$. Evaporation and recrystallization of the residue from *i*-Pr_2O afforded 4.24 g (74%) of **10** as a white solid. ^{1}H NMR (CD_3OD): 4.68 (m, 2 H); 2.49−2.32 (m, 4 H); 1.52 (s, 9 H). ^{13}C NMR (CD_3OD): 152.8, 118.7, 118.3, 83.0, 30.4, 29.5, 27.2. APCI MS: 222 (MH^+). mp: 113−114 °C. Anal. ($C_{11}H_{15}N_3O_2$) C, H, N.

General Procedure for the Preparation of Inhibitors 1: 1-({[1-(Hydroxymethyl)cyclopentyl]amino}acetyl)pyrrolidine-2,5-dicarbonitrile (1c). A solution of 0.149 g (0.615 mmol) of **2** in 2 mL of MeCN was added dropwise to a solution of 0.213 g (1.85 mmol) of 1-(hydroxymethyl)-1-cyclopentylamine in 4 mL of MeCN. The mixture was stirred for 48 h at room temperature, after which the mixture was evaporated. The residue was flash chromatographed on silica gel (eluting with 95:5 CH_2Cl_2−MeOH) to afford 0.120 g (70%) of **1c** as the free base, which was then taken up in 4 mL of MeOH, treated with 1 equiv of HCl in MeOH, and concentrated. The residue was taken up in MeCN, concentrated, and then crystallized from MeCN to afford 0.128 g (95%) of **1c** as the hydrochloride salt. ^{1}H NMR (D_2O): 5.04 (m, 1 H); 4.78 (m, 1 H); 4.14 (d, J = 16.6 Hz, 1 H); 4.04 (d, J = 16.6 Hz, 1 H); 2.54−2.35 (m, 4 H); 1.82−1.68 (m, 8 H); 1.62−15.4 (m, 2H). ^{13}C NMR (D_2O): 165.7, 118.0, 117.5, 70.9, 62.9, 47.6, 47.4, 44.4, 32.0, 30.9, 28.3, 24.0. APCI MS: 277 (MH^+). mp: 173−175 °C. Anal. ($C_{14}H_{20}N_4O_2$ HCl): C, H, N.

General Procedure for the Preparation of Tertiary Carbinamines from Tertiary Alcohols Using $TMSN_3$ and BF_3: 1-Methylcyclopentylamine Hydrochloride (19, amine for 1z). A solution of 2.00 g (20.0 mmol) of 1-methylcyclopentanol[26] in 25 mL of benzene was treated with 3.18 mL (23.9 mmol) of azidotrimethylsilane and 3.04 mL (24.0 mmol) of boron trifluoride etherate. After 24 h, the solution was poured into 50 mL of 1 M sodium bicarbonate solution and stirred for 30 min. Solid sodium bicarbonate was added as needed to maintain pH > 7. The benzene layer was separated and dried over anhydrous $CaCl_2$. The benzene solution was diluted with 25 mL of methanol and hydrogenated over 0.45 g of 10% Pd/C catalyst for 90 min at 45 psi and 20 °C. The reaction mixture was then filtered through diatomaceous earth, 1.7 mL of 12 M hydrochloric acid was added to the filtrate, and the solvent was evaporated to provide 1.92 g (71%) of 1-methylcyclopentylamine hydrochloride **19** as a white solid. ^{1}H NMR (D_2O): 1.59 (m, 8 H); 1.22 (s, 3 H). ^{13}C NMR (D_2O): 61.4, 37.7, 24.6, 23.7. APCI MS: 100 (MH^+). mp: 261−262 °C. Anal. ($C_6H_{13}N$ HCl): C, H, N.

General Procedure for the Preparation of Tertiary Carbinamines from N-Cbz-1-(hydroxymethyl)-1-cyclopentylamine: [1-(Propoxymethyl)cyclopentyl]amine (13y, amine for 1y). A solution of 11.5 g (99.8 mmol) of 1-(hydroxymethyl)-1-cyclopentylamine in 50 mL of CH_2Cl_2 was added over 30 min to a solution of 7.1 mL (50 mmol) of Cbz-Cl in 100 mL of CH_2Cl_2 with cooling in ice.[27] Stirring at 0 °C was continued for 30 min, and the mixture was filtered and concentrated to provide 12.48 g (100%) of **11** as a white solid. ^{1}H NMR ($CDCl_3$): 7.41 (m, 5 H); 5.11 (s, 2 H); 5.04 (br s, 1 H); 3.73 (s, 2 H); 1.87−1.66 (m, 8 H). APCI MS: 250 (MH^+). mp: 56−57 °C (from hexane). A mixture of **11** (4.98 g, 20 mmol) and freshly prepared Ag_2O (9.27 g, 40.0 mmol) in 15 mL of DMF was treated with allyl bromide (12 mL, excess) and stirred overnight at 20 °C. The reaction mixture was filtered and evaporated, and the residue was chromatographed on silica gel (eluting with 9:1 hexanes−EtOAc) to provide 5.31 g (91%) of **12** (R = allyl) as an oil. ^{1}H NMR ($CDCl_3$): 7.36−7.27 (m, 5 H); 5.84 (m, 1 H); 5.23 (d of d, J = 17.0 Hz, 1.6 Hz, 1 H); 5.14 (d of d, J = 10.4 Hz, 1.5 Hz, 1 H); 5.04 (br d, 2 H); 3.95 (m, 2 H); 1.92−1.88 (m, 2 H); 1.75−1.66 (m, 4 H); 1.58−1.52 (m, 2 H). APCI MS: 290 (MH^+). A mixture of 2.80 g (9.67 mmol) of **12** (R = allyl) and 0.80 g of 10% Pd on C was shaken under 50 psi of hydrogen overnight at 20 °C. The mixture was filtered and concentrated to provide 1.52 g (98%) of [1-(propoxymethyl)-cyclopentyl]amine (**13y**, R = propyl) as a clear liquid. ^{1}H NMR (CD_3OD): 3.42 (t, 2 H); 3.27 (s, 2 H); 1.78−1.45 (m, 8 H); 0.93 (t, 3 H). ^{13}C NMR ($CDCl_3$): 79.0, 73.4, 61.9, 37.8, 24.7, 23.0, 10.8. APCI MS: 158 (MH^+). Anal. ($C_9H_{19}NO$): C, H, N.

DPP-IV Enzyme Inhibition Screening Assay. An amount of 150 μL of an enzyme−substrate solution containing 50 μM Gly-Pro-4-methoxy-β-naphthylamide hydrochloride in 50 mM pH 7.3 Tris buffer containing 0.1 M sodium chloride, 0.1% (v/v) Triton, and 50 μU/mL DPP-IV[29] was pipetted into microtiter wells of a polystyrene 96-well plate maintained at 4 °C. Five microliters per well of test compound was added, bringing the final test compound concentrations to 3 μM to 10 nM per well. Diprotin A[28] (100 μM) was used as a positive control. The entire assay is incubated 18 h at 37 °C and then stopped by adding 10 μL of a solution containing 0.5 mg/mL Fast Blue B in a buffer comprising 0.1 M pH 4.2 sodium acetate and 10% (v/v) Triton X-100 to each well, followed by shaking for approximately 5 min at room temperature, after which the absorption maximum at 525 nm was determined. IC_{50} determinations were made from dose−response curves determined in triplicate. The reported IC_{50} values are the mean of at least three such determinations.

Plasma Enzyme Inhibition Assay. Aliquots (12.5 μL) of plasma were loaded onto a 96-well plate, and 10 μL of buffer pH 7.5 100 mM HEPES containing 0.2 mg mL^{-1} of BSA was added to each aliquot. The plate was briefly mixed on a titer plate shaker, sealed with plate sealing tape, and then placed in an incubator at 37 °C for 5 min. The reaction was initiated by the addition of 2.5 μL of substrate solution (500 μM H-Gly-Pro-AMC in buffer) to the wells, and the plate was shaken briefly. The plate was then read over a 5 min period in a Wallac Victor^2 1420 Multilabel Counter, using an excitation wavelength of 360 nm and recording the emission at 460 nm. IC_{50} determinations were made from dose−response curves determined in triplicate. The reported IC_{50} values are the mean of at least three such determinations.

KK/H1J Mouse Oral Glucose Tolerance Test. KK/H1J mice were fasted overnight with free access to water. After fasting, (time "*t*" = 0), 25 mL of blood was drawn from the retro-orbital sinus and added to 0.025% heparinized saline (100 mL) on ice. The mice (10 per group) were then orally dosed with a solution of a test compound in 0.5% methylcellulose (0.2 mL per mouse). Two controls groups received only 0.5% methylcellulose. At t = 15 min, the mice were bled, as described above, and then dosed with 1 mg kg^{-1} glucose in distilled water (0.2 mL per mouse). The first control group was dosed with glucose. The second control group was dosed with water. At t = 45 min, the mice were again bled as described above. The blood samples were centrifuged, and the plasma was collected and analyzed for glucose content. Data were expressed as percent inhibition of glucose excursion relative to the two control groups.[30]

Rat PK Determination. Male Sprague−Dawley rats (200−250 g) implanted with jugular vein cannulas (JVC) were obtained from Charles River Laboratories. Test compounds were administered as either an intravenous bolus via the JVC or by oral gavage. The intravenous dose was administered as a solution in sterile saline at 1.0 mg/kg and a dose volume of 1.0 mL/kg. The oral dose was administered as a solution in 0.5% methycellulose at 5.0 mg/kg and a dose volume of 10 mL/kg. Blood samples (0.25 mL) were collected at multiple time points from 0 to 24 h and placed into tubes containing lithium heparin. The blood samples were then centrifuged at 12000 rpm for 10 min. The plasma was removed and divided into two aliquots, one for determination of test compound plasma concentrations (pharmacokinetic analysis) and the second for determination the percent DPP-IV inhibition (pharmacodynamic effect). The plasma samples were frozen at −70 °C until analysis. The rat plasma samples were analyzed for test compound concentrations by LC/MS/MS using an Applied Biosystems API 4000 mass spectrometer. Briefly, test compound standard curves were prepared in control rat plasma with a dynamic range of 1.0−2000 ng/mL. Aliquots (0.02 mL) of both standards and samples were placed into Marsh tubes in a 96-well block. Proteins were precipitated by addition of 0.1 mL acetonitrile containing 0.1 mg/mL of internal standard. The 96-well blocks were vortexed and then centrifuged at 3000 rpm for 5 min. The resulting

supernatant was removed and placed into a new 96-well block and taken to dryness at 50 °C under a nitrogen stream. Residues were reconstituted in mobile phase (60% 5 mM ammonium acetate and 40% acetonitrile). Aliquots (0.01 mL) were then injected onto the LC/MS/MS for analysis.

X-ray Crystallography. The protein construct contained residues 31−766 of DPP-IV and a hexa-his tag linked via a thrombin cleavage site. The protein was secreted from insect cells and purified through conventional chromatography. The purified protein was then enzymatically deglycosylated and set up for crystallization. The crystal diffracted 2.6 Å resolution at APS 17ID. The crystal belonged to space group $P4_32_12$ with a = b = 69.1 Å and c = 410.8 Å. The crystal mosaicity was 0.5°. The data were 96.3% complete to 2.6 Å resolution with an R_{merge} of 0.109. There were 149578 reflections and 30926 unique reflections, giving an average redundancy of 4.8. The structure was solved using difference Fourier methods with the program AMORE.[31] The structure was refined with 2 iterations of REFMAC refinement and manual model building. The final R factor was 0.217 (R_{free} 0.281). The final model contained residues 31−766, and additionally contained eight *N*-acetylglucosamine (NAG) residues on six sites (85 × 2, 150 × 2, 219, 229, 281, 520), one inhibitor molecule **1c** and 179 water molecules.

Supporting Information Available: Elemental analyses for compounds **1a**−**z**, **1aa**−**gg**, **2**, **3**, **6**−**10**, **13y**, **14a**, **14b**, **15**−**17**, and **19**, observed APCI MS MH^+ ions for compounds in Tables 1 and 2, and structures of additional inactive (IC_{50} > 500 nM) *cis*-2,5-dicyanopyrrolidine inhibitors not presented in Tables 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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(1) Fact Sheet No. 138. World Health Organization, Geneva, 2002.

(2) For a recent review, see: Knudsen, L. B. Glucagon-like Peptide-1: The Basis of a New Class of Treatment for Type 2 Diabetes. *J. Med. Chem*. **2004**, *47*, 4128−4134.

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