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Discovery of non-covalent dipeptidyl peptidase IV inhibitors which induce a conformational change in the active site

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Abstract—A series of non-covalent inhibitors of the serine protease dipeptidyl peptidase IV (DPP-IV) were found to adopt a U-shaped binding conformation in X-ray co-crystallization studies. Remarkably, Tyr547 undergoes a 70° side-chain rotation to accommodate the inhibitor and allows access to a previously unexposed area of the protein backbone for hydrogen bonding.
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Dipeptidyl peptidase IV (DPP-IV) is a serine protease expressed pervasively throughout the human body. While mostly membrane bound, a small percentage of unbound DPP-IV can be found freely circulating in plasma.[1] Although DPP-IV is hypothesized to regulate many bioactive peptides, it is known that its major function is the truncation of glucagon-like peptide 1 (GLP-1), an incretin hormone which stimulates pancreatic insulin secretion.[2] Inhibition of DPP-IV has been shown to increase levels of GLP-1 in vivo resulting in improved control of glucose homeostasis in animal models of diabetes.[3] As such, the search for small molecule inhibitors of DPP-IV has been an area of intensive research across the pharmaceutical industry.[4]

Protease inhibitors traditionally rely upon warhead motifs for tight binding to the desired target.[5] From the emerging literature over the past decade, it is now appreciated that inhibitors can be found that do not require reactive functionalities or non-drug like features to achieve satisfactory levels of binding affinity. Serine protease research will likely continue to evolve away from synthesis of warhead based inhibitors as the hurdle of achieving selectivity is lowered when the key binding interactions do not rely on covalent modification of the enzyme. It is clear that there is still room for the medicinal chemistry community to improve the level of sophistication with respect to knowledge-guided identification of non-warhead containing serine protease inhibitors.[6] Herein we describe a series of DPP-IV inhibitors that engage the S1 pocket by utilizing a mildly electrophilic aryl ketone which, unexpectedly, does not engage the catalytic serine in traditional warhead fashion. Additionally, the unique binding mode of this scaffold forces a conformational movement in one of the active site residues to provide a series of highly potent and selective non-covalent inhibitors.

Prosecution of a high throughput screen resulted in the identification of the secondary amine containing compound **1**, which demonstrated an IC_{50} = 84 nM (Fig. 1).[7]

Keywords: Dipeptidyl peptidase IV; Protease inhibitor; X-ray co-crystal structure; Oral glucose tolerance test.
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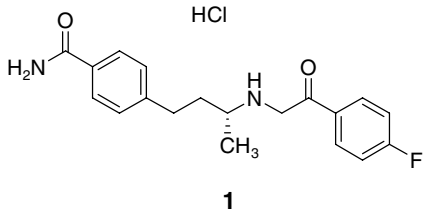


Figure 1. An aryl ketone derived non-covalent inhibitor of DPP-IV.

An X-ray co-crystal structure of **1** bound in DPP-IV revealed an unanticipated binding mode. The inhibitor adopts a U-shaped conformation which is accommodated predominately by a side-chain positional shift of Tyr547. Specifically, the Tyr547 χ^1 dihedral angle changes by 70° compared to previous observations (Fig. 2).[8] The phenyl carboxamide π system in **1** is offset from, but parallel to the phenyl ring of Tyr547, with the two ring systems being approximately 3.4 Å apart. Importantly, the carbonyl of the primary carboxamide is located within hydrogen bonding distance to the protein backbone NH of Tyr631. The aryl ketone moiety occupies the S1 pocket with the lone pair of electrons on the carbonyl oxygen available to interact with Asn710 to form a hydrogen bond. The secondary amine is likewise within hydrogen bonding distance to Glu206. Furthermore, this U-shaped ligand conformation was calculated to be approximately 3 kcal/mol lower in energy compared to a fully extended conformation.[9] This suggests that the U-shaped ligand form is likely pre-folded, and there is no energetic penalty for adopting it.

Inhibitors of this type can be readily prepared by the microwave-assisted alkylation of α-branched amines with 2-bromoacetophenone derivatives (Scheme 1). For example, irradiation of 4-((*R*)-3-amino-butyl)-benzonitrile **2** and 2-bromo-4′-fluoroacetophenone **3** for 15 min at 100 °C followed by filtration of the resultant precipitate provides intermediate **4** in 59% yield.[11] Hydrolysis of the cyano moiety with hydrochloric acid

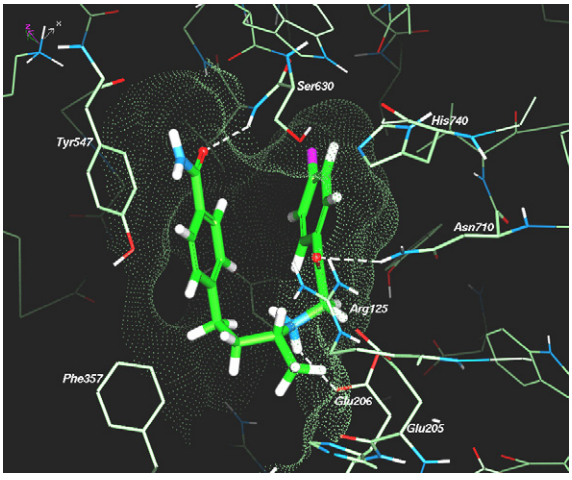
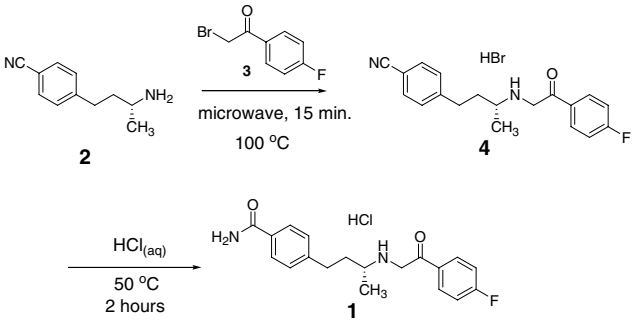


Figure 2. 2.1 Å resolution X-ray co-crystal structure of compound **1** bound in DPP-IV.[10]



Scheme 1. Representative preparation of aryl ketone DPP-IV inhibitors.

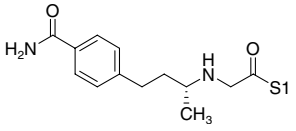
followed by filtration of the resultant precipitate provides **1** in 92% yield.[12]

The SAR is consistent with the binding mode from the X-ray structure which places the phenyl ring of the aryl ketone into the well-defined hydrophobic S1 pocket (Table 1). In particular, fluorine located in the para-position of a phenyl S1 substituent provides a 3× boost in activity relative to the unsubstituted phenyl analog **5**, while the larger chloro and methyl substituents at the same position are less tolerated as seen in compounds **8** and **9**. Fluorine substitution at the ortho-position as demonstrated by compound **7** provides a decrease in activity. Disubstitution with fluorine at the meta- and para-positions (compound **6**) provides a marginal improvement in activity relative to monofluoro para-substitution (compound **1**). Interestingly, the 3-thiophene S1 binder of compound **10** demonstrates equivalent activity to the 4-fluorophenyl S1 binder.

Focused SAR directed toward optimization of the linkage between the phenyl carboxamide and the α-amino ketone portion of the molecule resulted in the discovery of several active molecules, highlighted by inhibitors **11** and **12** (Fig. 3).[14]

The X-ray co-crystal structures of **10** and **12** in DPP-IV confirmed a similar U-shaped binding conformation as was found for **1**. Despite this similarity, the structures do reveal some differences in the placement and engage-

Table 1. S1 binders



Compound	S1	DPP-IV IC_{50}[6] (nM)
5	Phenyl	280
1	4-Fluorophenyl	84
6	3,4-Difluorophenyl	39
7	2-Fluorophenyl	3800
8	4-Chlorophenyl	820
9	4-Tolyl	21,000[a]
10	3-Thiophene	110

[a] Compound **9** was tested as a racemate.[13]

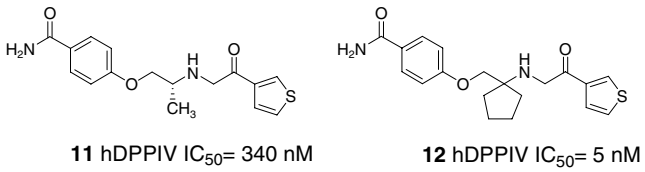


Figure 3. Ether based linkers for α-amino ketone non-covalent inhibitors of DPP-IV.

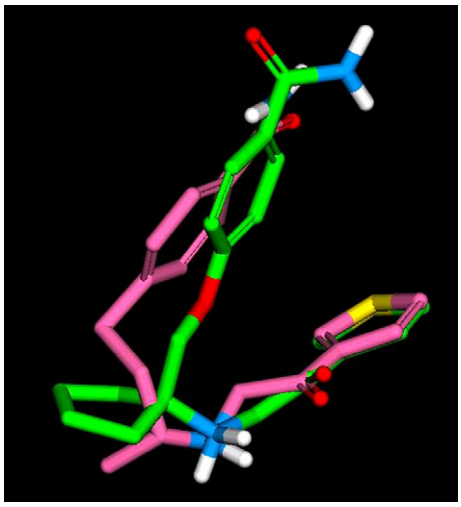


Figure 4. Overlay of co-crystal structure of compound **10** (in pink) and compound **12** (in green) bound in DPP-IV with the view of the protein hidden for clarity.

ment of the ligands in the newly formed binding region above the S1 pocket. Figure 4 shows the ligands superimposed from DPP-IV co-crystal structures of the thiophenyl ketone comparator compound **10** (in pink) and compound **12** (in green), with the proteins removed for clarity. As seen here, modification from the methyl branched propyl linker in **10** to the 1,1-disubstituted cyclopentyl ethyl ether linker in **12** does not result in significant differences in binding position within the S1 pocket (as illustrated by the thiophene rings), but does appear to result in a different point of engagement of the protein backbone by the carboxamide moiety (Fig. 4). The structures suggest that the amide carbonyl in compound **10**, which like compound **1** contains the methyl branched alkyl linker between the amine and phenyl ring, engages in hydrogen bonding with the NH of Tyr631. Alternatively, the constraint provided by the cyclopentyl ether linkage in **12** pushes the aryl carboxamide higher into the newly formed region and allows opportunity for hydrogen bonding between the carboxamide and the carbonyl of Trp629. Also noteworthy is that the carboxamide moiety in **12** appears to be rotated out of the plane of the phenyl ring in order to obtain the putative hydrogen bond with Trp629.

Oral administration of 30 mpk of compound **12** produced a modest, yet significant, glucose lowering effect (24% reduction relative to vehicle alone) in an oral glucose tolerance test (OGTT) in healthy rats.[15] This was

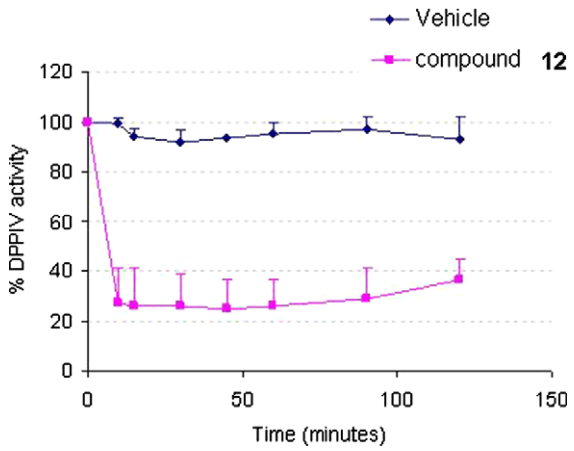


Figure 5. Ex vivo DPP-IV inhibition after a 30 mpk oral administration to healthy rats.

consistent with the level of ex vivo DPP-IV inhibition measured over a 2 h time period (Fig. 5).

Profiling of compound **12** against a broad receptor panel, related proteases as well as cytochrome P450 enzymes revealed no significant cross reactivity.[16]

In conclusion, a series of selective DPP-IV inhibitors was discovered to have a novel U-shaped mode of non-covalent binding to the active site. This new binding motif is most notably defined by the displacement of Tyr547 to allow inhibitors to engage in hydrogen bonding with either Tyr631 or Trp629. Inhibitors of this class, exemplified by compound **12**, demonstrate activity in vivo in healthy rats as demonstrated by an OGTT and this activity is consistent with the pharmacodynamic activity of this compound over a similar time course. Additional profiling and further improvements to these molecules will be reported in due course.

Acknowledgments

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7. (a) IC_{50} values reported in this manuscript are means of at least two separate experiments. The DPP-IV assay uses a fluorometric end point assay (excitation 355 nm; emission 460 nm), enriched human recombinant DPP-IV enzyme (21.3 μU/μL), and Gly-Pro-AMC (Bachem I-1225) as substrate (0.02 mM). Secreted DPP-IV (lacking membrane anchor) is enriched from HEK293 cell culture supernatant by ultra-filtration, ultra-centrifugation, and size-exclusion chromatography. IC_{50} values of the compounds are calculated based on a 12-point concentration response curve with each concentration measured in duplicate. The assay is validated by plate variability and conformity, inter-plate variability, signal window, and minimum significant ratio (MSR) of IC_{50}. A MSR is calculated based on a test/retest analysis and a retrospective analysis. Using the statistical method of Bland and Altman, IC_{50} data generated in this fashion have been determined to have a minimum significant ratio of 1.5; (b) Bland, J. M.; Altman, D. G. *Lancet* **1986**, *1*, 307.
8. (a) Rasmussen, H. B.; Branner, S.; Wiberg, F. C.; Wagtmann, N. *Nat. Struct. Biol.* **2003**, *10*, 19; Recently, displacement of Tyr547 by a dihydro-purine derived DPP-IV inhibitor has been disclosed (b) Engel, M.; Hoffmann, T.; Manhart, S.; Heiser, U.; Chambre, S.; Huber, R.; Demuth, H.-U.; Bode, W. *J. Mol. Biol.* **2006**, *355*, 768; Recently, inhibitor induced movement of Tyr548 in rat DPP-IV has been disclosed (c) Longenecker, K. L.; Stewart, K. D.; Madar, D. J.; Jakob, C. G.; Fry, E. H.; Wilk, S.; Lin, C. W.; Ballaron, S. J.; Stashko, M. A.; Lubben, T. H.; Yong, H.; Pireh, D.; Pei, Z.; Basha, F.; Wiedeman, P. E.; von Geldern, T. W.; Trevillyan, J. M.; Stol, V. S. *Biochemistry* **2006**, *45*, 7474.
9. Energy minimization of the X-ray bound and linear conformations of **1** was performed with CHARMm (QUANTA2005, Accelrys Software Inc.) using no non-bond cutoffs, a distance dependent dielectric, and a 3.0 dielectric constant. Conformational energy differences (linear–U-shaped) were found to be 3.6 (CHARMm) and 2.8 (MMFF) kcal/mol for each force field, respectively.
10. (a) Coordinates for structure **1** have been deposited with the RCSB protein databank. PDB deposition #20GZ; (b) DPPIV crystals were grown in the presence of compounds **1**, **11**, and **12** using methods described by Rasmussen, H. B.; Branner, S.; Wiberg, F. C.; Wagtman, N. R. *Nat. Struct. Biol.*, **2003**, *10*, 19. Diffraction data were collected at 100 K at the Advanced Photon Source sector 17 (IMCA-CAT). Structures of DPPIV complexed with compounds **1**, **11**, and **12** were solved using models initially derived from 1N1M.pdb and were refined to resolutions of 2.1, 2.0, and 2.3 Å, respectively.
11. Preparation of **4**: To a 10 ml pyrex microwave reaction vessel was added a solution of 4-((*R*)-3-amino-butyl)-benzonitrile**2** (383 mg, 2.20 mmol) in acetonitrile (6 ml) followed by addition of 2-bromo-4′-fluoroacetophenone **3** (572 mg, 2.64 mmol). The reaction vessel was capped, and the reaction was immediately subjected to microwave irradiation for 20 min at 100 °C (60 W). The vessel was allowed to cool at which time the product crystallized. The solid was collected by filtration and washed with acetonitrile:ethyl ether (1:1) to afford 512 mg of product as a white crystalline solid. ^{1}H NMR (400 MHz, CD_3OD) δ 1.48 (d, 3H, J = 6.4 Hz), 1.89–1.98 (m, 1H), 2.17–2.27 (m, 1H), 2.76–2.86 (m, 1H), 2.89–2.98 (m, 1H), 3.35–3.44 (m, 1H), 7.32 (dd, 1H, J = 8.8 and 9.2 Hz), 7.48 (d, 1H, J = 8.4 Hz), 7.68 (d, 1H, J = 8.4 Hz), 8.16–8.12 (m, 1H); ^{13}C NMR (400 MHz, CD_3OD) 14.9, 31.2, 33.8, 49.8, 54.1, 109.8, 115.7, 115.7, 118.4, 129.3, 130.2, 131.2, 132.1, 146.5, 165.4, 167.9, and 190.2. Mp 215 °C. HRMS calcd for $C_{19}H_{19}FN_2O$ [M+H] 311.1515; found: 311.1553.
12. Preparation of **1**: A solution of **4** (600 mg, 1.53 mmol) in con. HCl (10 ml) was heated to 50 °C for 5.5 h. The reaction mixture was cooled and ice was added. The resulting solid was collected by filtration and washed with water to afford 515 mg of product as a white crystalline solid. ^{1}H NMR (400 MHz, CD_3OD) δ 1.50 (d, 3H, J = 6.8 Hz), 1.92–2.30 (m, 1H), 2.21–2.30 (m, 1H), 2.71–2.86 (m, 1H), 2.90–2.99 (m, 1H), 3.37–3.45 (m, 1H), 4.80 (s, 2H), 7.35 (dd, 1H, J = 8.8 and 9.2 Hz), 7.42 (d, 1H, J = 8.8 Hz), 7.87 (d, 1H, J = 8.8 Hz), 8.15–8.20 (m, 1H); ^{13}C NMR (400 MHz, CD_3OD) δ 14.9, 31.0, 34.1, 49.7, 54.2, 115.7, 115.9, 127.8, 128.3, 130.2, 131.1, 145.1, 165.4, 167.9, 171.0, and 190.2. ^{13}C NMR (400 MHz, CD_3OD) 14.9, 31.2, 33.8, 49.8, 54.1, 109.8, 115.7, 115.7, 118.4, 129.3, 130.2, 131.2, 132.1, 146.5, 165.4, 167.9, and 190.2. Mp 205 °C. HRMS calcd for $C_{19}H_{21}FN_2O_2$ [M+H] 329.1621; found: 329.1660.
13. We have found that the majority of the DPP-IV activity results from a single enantiomer (unpublished results) and as such occasionally racemates are tested for expedience.
14. The intermediate amines used in the synthesis of **11** and **12** were prepared via S_NAr reaction of the corresponding commercially available amino alcohol with 4-fluorobenzonitrile following the procedure of Vogler, M.; Koert, U.; Harms, K.; Dorsch, D.; Gleitz, J.; Raddatz, P. *Synthesis*, **2004**, *8*, 1211.
15. OGTT experiment: the compound was administered orally to fasted male Wistar rats (n = 6) 10 min before a glucose load of 2.5 g/kg. Blood samples were collected from the tail tip over 120 min for the determination of glucose (hexokinase method), insulin (immunoassay from CrystalChem Inc., ref. INSKR20), and DPP-IV activity (fluorimetric endpoint assay using Gly-Pro-AMC (Bachem I-1225) as substrate and AMC (Bachem Q-1025) for calibration).
16. Compound **12** was found to have >10,000× selectivity over dipeptidyl peptidases 8 and 9.