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Inhibition of dipeptidyl peptidase-IV (DPP-IV) by atorvastatin

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Abstract—Dipeptidyl peptidase-IV (DPP-IV) is an enzyme responsible for the inactivation of the glucoregulatory incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). In this report, we show that the hypolipidemic agent atorvastatin is a competitive inhibitor of porcine DPP-IV in vitro, with $K_i = 57.8 \pm 2.3$ μM. These results may have implications in the development of novel DPP-IV inhibitors based on the use of atorvastatin as a lead compound for the treatment of type 2 diabetes.

Dipeptidyl peptidase-IV (DPP-IV) [EC 3.4.14.5] is a serine protease responsible for the degradation of a number of biological peptides including glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). GLP-1 and GIP are incretins released from the gut in response to food and play an essential role in maintaining glucose homeostasis.[1,2] Together, they are responsible for up to 70% of insulin secreted following a meal.[3] However, the effects of these incretins in type 2 diabetics are severely diminished[4] and inhibition of DPP-IV has been shown to improve glucose tolerance in these patients by enhancing the insulinotropic effects of GLP-1.[5] As a result, the search for inhibitors of DPP-IV for the treatment of type 2 diabetes is an active area of research.[6,7]

Three general classes of DPP-IV inhibitors with in vivo efficacy can be described:[7] (1) reversible substrate analogs; (2) covalently modifying substrate analogs; (3) reversible non-peptide heterocyclic compounds. A variety of structures are known, but it seems that a requirement for all inhibitors is a basic amine at the P_2-position.[6] Both the reversible and covalently modifying substrate analog inhibitors take advantage of the high preference of DPP-IV for proline binding within the S_1 hydrophobic pocket. These inhibitors are generally amide derivatives of pyrrolidine (α-aminoacylpyrrolidines) or thiazolidine (α-aminoacylthiazolidines), which bind with even greater affinity. Examples include valine-pyrrolidide[8] and isoleucine-thiazolidide[9] (Fig. 1). The covalently modifying inhibitors contain in addition an electrophilic group at the 2-position of the pyrrolidine or thiazolidine ring and are even more potent. The most common group is cyano, which is capable of forming an enzyme-imidate adduct with the active site Ser630 and is present in vildagliptin[10] and saxagliptin[11] (Fig. 1).

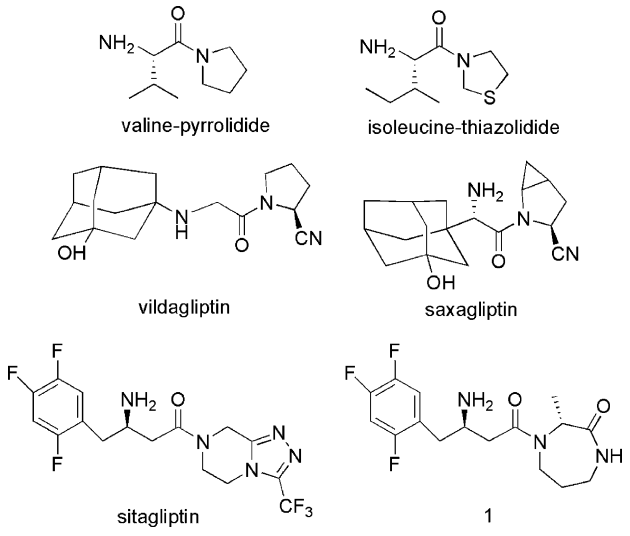

Figure 1. Structures of different classes of DPP-IV inhibitors.

Keywords: Diabetes; Statin; Molecular modeling.
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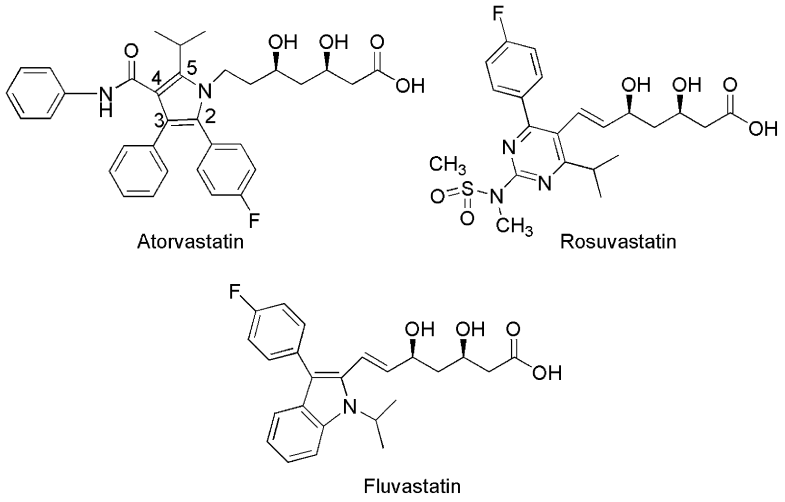

Figure 2. Structures of the statins examined in this study.

The reversible non-peptide heterocyclic inhibitors include sitagliptin[12] (Fig. 1), which has recently obtained FDA approval (October 2006), (3*R*)-4-[(3*R*)-3-amino-4-(2,4,5-trifluorophenyl)butanoyl]-3-(methyl)-1,4-diazepan-2-one (**1**),[13] and other compounds from a diverse array of classes. These do not contain a pyrrolidine or thiazolidine ring to bind within the S_1 pocket. Instead, sitagliptin has a piperazine-pyrazole fused ring system and binds to the enzyme with the amide moiety in an orientation opposite to that of substrate analog inhibitors so that the 2,4,5-trifluorophenyl group binds within the S_1 hydrophobic pocket.[12] An amide bond is not even necessary for activity as xanthine, aminomethylpyrimidine, isoquinoline, and isoquinolone inhibitors are known.[7]

Several classes of antidiabetic drugs have recently been investigated for their effect on DPP-IV. Metformin has been reported to inhibit DPP-IV[14–16] with IC_{50} ranging from 29 to 98 μM, though others have refuted this claim.[17–19] Thiazolidinediones (rosiglitazone, troglitazone), sulfonylureas (glybenclamide, tolbutamide), and meglitinides (nateglinide) have also been reported to inhibit DPP-IV.[20,21] While hyperlipidemia is a complication of diabetes mellitus, to date no work has investigated hypolipidemic drugs such as the statins for concurrent DPP-IV inhibiting activity. Statins are competitive inhibitors of the enzyme hydroxymethylglutaryl-CoA (HMG-CoA) reductase, which catalyzes the rate-limiting step in cholesterol biosynthesis. One such statin (atorvastatin) has also been shown to be effective in preventing cardiovascular complications associated with type 2 diabetes.[22] Therefore, the use of statins in such patients is likely to become more prevalent.

In this study, three structurally related statins, atorvastatin, rosuvastatin, and fluvastatin (Fig. 2), were evaluated for their ability to inhibit DPP-IV catalyzed hydrolysis of the synthetic substrate Gly-Pro-*p*-nitroanilide in vitro at a concentration of 500 μM. We found that atorvastatin inhibited DPP-IV activity (66 ± 4%), whereas both rosuvastatin (4 ± 2%) and fluvastatin (8 ± 1%) had little effect. Since atorvastatin was the most active compound, we subjected it to further evaluation and determined its IC_{50} to be 175.1 ± 1.1 μM (Table 1). Diprotin A (Ile-Pro-Ile) is a known competitive inhibitor of DPP-IV with IC_{50} = 14.4 ± 0.4 μM (Table 1) and was used as control.

There are a number of compound properties that are generally considered undesirable, including the ability to form aggregates. Such aggregates can inhibit a variety of unrelated enzymes by absorption onto the surface of enzymes or by incorporating enzymes within them[23–25] and compounds acting in this manner are called 'promiscuous' inhibitors. Ryan et al.[26] have shown that with high detergent concentrations, about 0.5 critical micellar concentration (CMC), total suppression of promiscuous inhibition is possible. Therefore, in order to investigate the potential of atorvastatin behaving as a promiscuous inhibitor the effect of three detergents (Tween-20, Triton X-100, and cholic acid) at 0.5 CMC[27] on DPP-IV enzyme activity was determined. Interestingly, the IC_{50} value of atorvastatin against DPP-IV in the presence of any of these detergents remained unchanged (169–177 μM) compared to the IC_{50} value (175.1 ± 1.1 μM) determined in the absence of any of these detergents. These results suggest that atorvastatin does not inhibit DPP-IV by aggregation, but rather by a classical 1:1 mechanism.

DPP-IV catalyzed hydrolysis of Gly-Pro-*p*-nitroanilide was measured while simultaneously varying the concentrations of atorvastatin and substrate.[28] Lineweaver–Burk plot of the data shows that inhibition was competitive (Fig. 3) and when the data were fits globally to the untransformed equation for competitive inhibition, a K_i = 57.8 ± 2.3 μM was obtained. Atorvastatin does not structurally resemble any of the known DPP-IV inhibitors and, in particular, does not contain a sufficiently basic amino group to potentially interact with the enzyme. Therefore, docking studies were performed to further comprehend the molecular mechanism how atorvastatin binds to DPP-IV.

Table 1. Summary of biochemical and computational data

Compound	Isolation method[a]	Binding affinity		% Inhibition at 500 μM	IC_{50} (μM)
		Gscore[b]	Emodel[b]		
Atorvastatin	Silica gel	−8.36	−77.21	66 ± 4	175.1 ± 1.1
Rosuvastatin	Sephadex LH-20	−6.22	−60.00	4 ± 2	ND
Fluvastatin	Sephadex LH-20	−7.21	−60.85	8 ± 1	ND
Diprotin A	—	—	—	—	14.4 ± 0.4

ND, not determined.

[a] Each was obtained from their respective tablet by crushing to a fine powder and extracting with MeOH. Atorvastatin was isolated from the methanol extract by silica gel chromatography with 100% EtOAc followed by 90% EtOAc: 10% MeOH. Rosuvastatin and fluvastatin were isolated by filtering the methanol extract through an ODS (C-18) solid phase extraction filter followed by Sephadex LH-20 chromatography with 100% ethanol. Purity was determined by TLC and structures were verified by GC–MS. Diprotin A was obtained from Sigma (St. Louis, MO).

[b] A more negative Gscore/Emodel indicated a better fit in the catalytic site. Both of these scoring functions are expressed as kcal/mol.

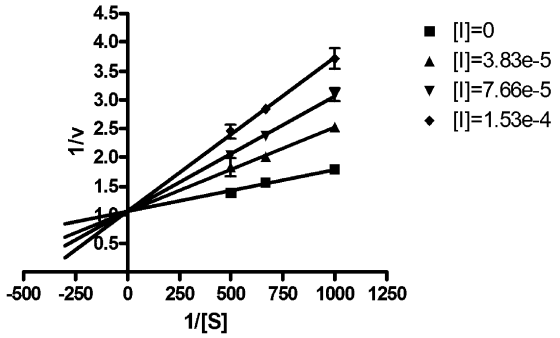

Figure 3. Lineweaver–Burk plot of atorvastatin and DPP-IV.

Using a recently solved DPP-IV co-crystal structure with the diazepanone inhibitor **1** (IC_{50} = 6.6 nM) as a template, atorvastatin, rosuvastatin, and fluvastatin were docked into the catalytic site of DPP-IV (PDB ID: 2iiv)[13] using the Glide 4.5 docking program.[29] Among several DPP-IV crystal structures bound with inhibitors we were prompted to use **1** as exemplified by its structural similarity with atorvastatin (Fig. 4A). The amide phenyl ring is closely mapped onto the 2,4,5-trifluorophenyl ring of **1**, whereas the flexible heptanoic acid moiety could potentially interact with Arg358 in the β-propeller domain, as discussed below. For docking experiments all the compounds were constructed using the fragment dictionary of Maestro 8.0 and geometry optimized using the Optimized Potentials for Liquid Simulations-all atom (OPLS-AA)[30] force field with the steepest descent followed by a truncated Newton conjugate gradient protocol as implemented in Macromodel v. 9.5. DPP-IV was optimized for docking using the protein preparation wizard provided by Schrodinger LLC and the Impact program (FirstDiscovery v4.5). Partial atomic charges for compounds as well as protein were assigned according to the OPLS-AA force field. The extra precision (XP) Glide docking method was then used to dock all compounds into the catalytic site of DPP-IV. Although details regarding the methodology used by Glide are described elsewhere,[31–34] a brief description is provided below. Grids for Glide docking were calculated using the bound inhibitor as the reference of catalytic site in the DPP-IV. Upon completion of each docking calculation, 50 poses per ligand were allowed to generate. The top-scored pose was chosen using a Glidescore (Gscore) function. The Gscore is a modified and extended version of the empirically based Chemscore function.[35] Another scoring function used by Glide is Emodel, which itself is derived from a combination of the Gscore, Coulombic, van der Waals, and the strain energy of the ligand. The docking methodology was validated by extracting the crystallographic bound diazepanone inhibitor **1** and redocking it to the catalytic site of DPP-IV. This validation provided a root mean square deviation (rmsd) of 0.168 Å between the docked versus the experimental conformation.

Human DPP-IV contains a catalytic domain formed by the residues Gln508-Pro766 and a β-propeller domain consisting of residues Lys56-Asn497. Docking studies show that atorvastatin binds to the active site of DPP-IV with the amide moiety in the opposite orientation to that reported for diazepanone **1**[13] (Fig. 4B). The *N*-phenyl moiety of the amide function was bound within the large S_1 hydrophobic cavity formed by Tyr547, Tyr631, Val656, Trp659, Tyr662, Tyr666, Val711, and His740 at the interface of the two domains close to two of the three catalytic residues (Ser630 and His740). A major contribution to binding by the *N*-phenyl moiety of the amide group is achieved by edge on pi–pi interaction with Tyr662 and edge to face pi–pi interaction with Tyr666. Substrate entry within this large cavity will not be possible because of the phenyl ring occupying the same pocket. Additionally, Tyr547 was found to establish hydrophobic interactions with the C-3 phenyl ring and Tyr666 was involved in hydrophobic contacts with the C-5 isopropyl group. The amide carbonyl oxygen atom is within hydrogen bonding distance (2.20 Å) to the guanidine side chain of Arg125, which in turn may be stabilized by an ionic interaction with Glu205. Crystallographic studies have shown that the negatively charged carboxyl terminus of diprotin A can interact with the guanidine group of Arg125.[36] The amide carbonyl oxygen can also establish additional long range hydrogen bonding interaction with the hydroxyl group of Tyr547 through crystallographic water molecule (#9118). The hydroxyl group of Tyr547 has been shown to stabilize the negatively charged oxyanion of the tetrahedral intermediate through a hydrogen bonding network.[36]

The fluorine on the C-2 phenyl ring does not make any contacts to the enzyme except perhaps weakly to

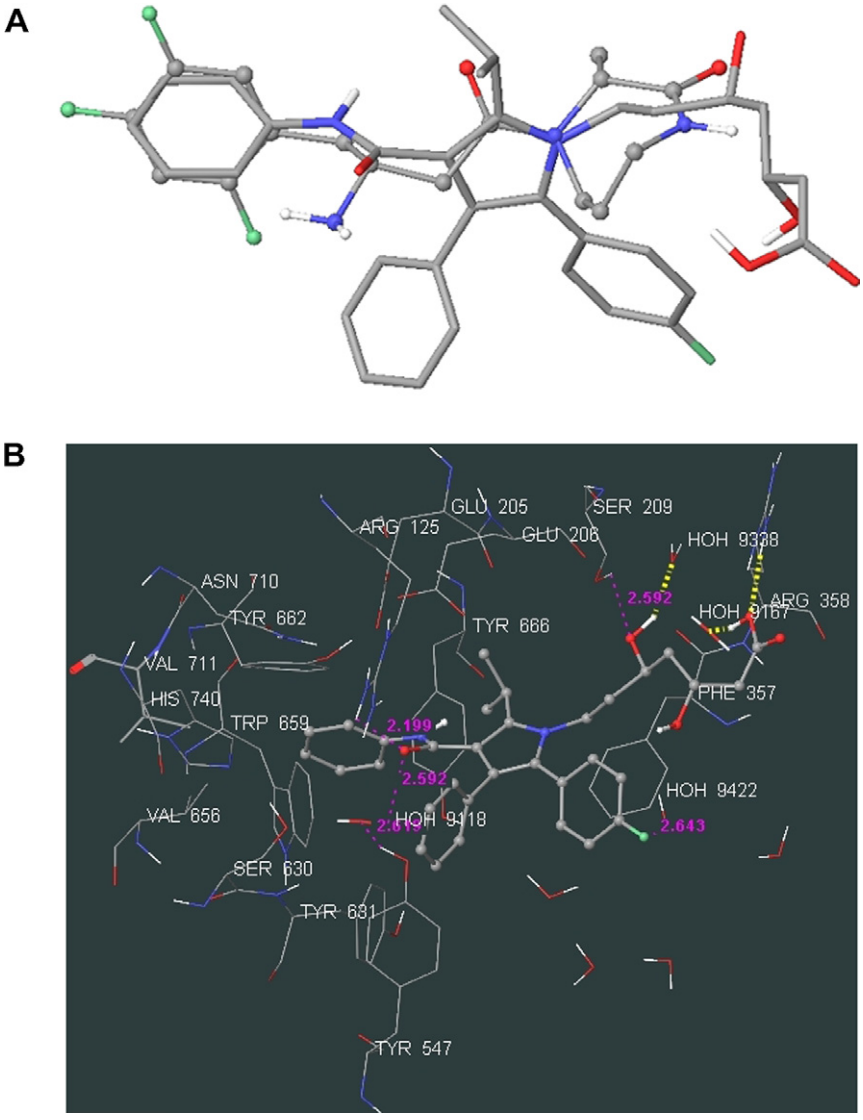

Figure 4. (A) Overlaid structures of diazepanone **1** (ball and stick) and atorvastatin (stick). (B) XPGlide-predicted pose for atorvastatin within the catalytic site of DPP-IV (PDB ID: 2iiv). For clarity, only polar hydrogen atoms are shown. Hydrogen bonds are shown as dotted yellow lines, whereas dotted pink lines indicate distance in Å. Catalytic site residues are represented as sticks with the atoms in standard color (i.e., carbon – gray, nitrogen – blue, oxygen – red and, hydrogen – white). Atorvastatin is shown as ball and stick model with same color scheme as above.

Phe357. This group is directed toward a large substrate access cavity that invariably must be filled with water molecules and as a result one can expect favorable entropy for inhibitor–enzyme complex formation. It is within hydrogen bonding distance to a water molecule (2.64 Å; #9422) and a probable role of the fluorophenyl moiety is displacement of several crystallographic water molecules found within a radius of 3-5 Å. Phe357 is involved in van der Waals or hydrophobic interactions with the C-6 and C-7 atoms of the heptanoic acid side chain. Whereas the amide phenyl and C-3 phenyl rings are engaged in strong hydrophobic interactions, the 3,5-dihydroxyheptanoic acid moiety forms a strong electrostatic network with crystallographic water molecules as well as with Ser209 and Arg358. The C-5 hydroxy group forms a hydrogen bond to a water molecule (–OH–OH_2-9338, 2.05 Å, 141.3°) and is within hydrogen bonding distance to the side chain of Ser209 (2.59 Å) within the β-propeller domain. The carboxyl group forms hydrogen bonds with the side chain of Arg358 (O—H_2N-Arg358, 2.04 Å, 174.9°) and with a water molecule (–COOH—OH_2-9167, 2.0 Å, 154.8°). It does not appear that the pyrrole ring directly interacts with the enzyme. Rather, it is likely that it only serves as a structural scaffold to properly orient the hydrophobic groups on one side and the polar groups on the other side for better binding.

In contrast to inhibitors containing a basic amino group (Fig. 1) there is no possibility for a direct ionic interaction with Glu205 or Glu206. The presence of such a basic amino group has been claimed as a structural prerequisite for inhibition.[6] Atorvastatin is likely stabilized by its interactions with water molecules; both by directly binding to them with its polar groups and by displacing them through the hydrophobic region of the molecule.

The binding mode of atorvastatin suggests ways to further improve upon its potency. Substitution of the 2,3,4-position of the amide phenyl ring with H-bond acceptor or donors may lead to greater binding. 3,4-substituents should interact with the side chain of Ser630 and the backbone of Tyr631 of the nucleophilic elbow, while the 2-substituent should interact with the side chain of Asn710. As has already been mentioned the amide phenyl ring occupies the large S_1 hydrophobic pocket. There is more room in this pocket and to better fill it a linker of 1–2 carbons may be added connecting the amide carbonyl to the pyrrole ring. Based on the docking model the isopropyl group methyls are 3–4 Å from the double Glu motif (Glu205/206) side chain carboxyl function. Substitution with $-NH_2$ or $-CH_2NH_2$ group could lead to greater interaction. The double Glu motif has been shown to bind to the positively charged amine of diprotin A.[36]

In contrast to atorvastatin, all of the poses for fluvastatin and rosuvastatin show that the polar 3,5-dihydroxyheptanoic acid moiety occupies the S_1 pocket and forms only one hydrogen bond (with Glu205 and Glu206, respectively). It is known that bulky or hydrophilic groups are poorly tolerated by the S_1 pocket.[36] In addition, both rosuvastatin and fluvastatin lack the hydrophobic contacts to Tyr547 that atorvastatin possesses with its C-3 phenyl.

From these studies, it appears as if inhibition is structure specific and therefore not every statin can be expected to bind to and inhibit DPP-IV. Any structure optimization should start with atorvastatin as a lead molecule. The results described in this paper may be useful in the design and development of novel statin-like DPP-IV inhibitors.

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incubation period of 15 min at 37 °C, 25 μl of 25% acetic acid (in methanol) was added to each well to halt the reaction and absorbance at 405 nm was determined using a microplate reader. The assay was performed in duplicate three times and with appropriate blanks and controls. All concentrations refer to final assay values. The data were analyzed with GraphPad Prism version 4 by globally fitting it to the untransformed equations for competitive, noncompetitive, and uncompetitive inhibition.

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