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Inhibition of dipeptidyl peptidase IV and xanthine oxidase by amino acids and dipeptides

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Abstract

Xanthine oxidase (XO) and dipeptidyl peptidase IV (DPP-IV) inhibition by amino acids and dipeptides was studied. Trp and Trp-containing dipeptides (Arg-Trp, Trp-Val, Val-Trp, Lys-Trp and Ile-Trp) inhibited XO. Three amino acids (Met, Leu and Trp) and eight dipeptides (Phe-Leu, Trp-Val, His-Leu, Glu-Lys, Ala-Leu, Val-Ala, Ser-Leu and Gly-Leu) inhibited DPP-IV. Trp and Trp-Val were multifunctional inhibitors of XO and DPP-IV. Lineweaver and Burk analysis showed that Trp was a non-competitive inhibitor of XO and a competitive inhibitor of DPP-IV. Molecular docking with Autodock Vina was used to better understand the interaction of the peptides with the active site of the enzyme. Because of the non-competitive inhibition observed, docking of Trp-Val to the secondary binding sites of XO and DPP-IV is required. Trp-Val was predicted to be intestinally neutral (between 25 and 75 % peptide remaining after 60 min simulated intestinal digestion). These results are of significance for the reduction of reactive oxygen species (ROS) and the increase of the half-life of incretins by food-derived peptides.

Key words: dipeptidyl peptidase IV inhibitors, xanthine oxidase inhibitors, amino acids, dipeptides, milk, predictive modeling, AutoDock Vina, intestinal stability

1. Introduction

Various potential health promoting and disease risk reducing activities for milk proteins and milk-derived peptides have been reported (FitzGerald & Meisel, 2003; Pihlanto, 2006). Insulin secretion from pancreatic beta cells in the presence of glucose is influenced by incretins such as glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Enzymatic degradation of these hormones by dipeptidyl peptidase IV (DDP-IV) activity can significantly reduce their level *in vivo* (Guasch, Ojeda, González-Abuín, Sala, Cereto-Massagué, Mulero, et al., 2012). It has been reported that peptides with 2-8 amino acid containing hydrophobic amino acid residues including Pro residues can inhibit DPP-IV. It has also been shown that milk derived peptides can inhibit DPP-IV activity (Lacroix & Li-Chan, 2012a, 2012b; Nongonierma & FitzGerald, 2013). Food protein derived DDP-IV inhibitory peptides may play a role in type 2 diabetes management by increasing the half-life of GIP and GLP-1 (Lacroix & Li-Chan, 2012a).

Cardiovascular and renal disease are complications associated with type 2 diabetes and insulin resistance syndrome. A link between hyperuricemia, the development of atherosclerosis, hypertension and insulin resistance has been proposed (Hayden & Tyagi, 2001). In type 2 diabetes patients, the enzyme-based antioxidant systems involving superoxide dismutase, glutathione peroxidase and catalase may be depleted arising from increased oxidative stress (Hayden & Tyagi, 2001). An increase in the concentration of reactive oxygen species (ROS) in endothelial cells can result in endothelial injury. Supplementation of anti-oxidants through dietary intake has been proposed as a means to counteract oxidative stress. The *in vitro* anti-oxidative properties of milk proteins and peptides have been reviewed (Pihlanto, 2006; Power, Jakeman, & FitzGerald, 2012). An increase in the concentration of ROS can occur during the oxidation of xanthine to uric acid catalyzed by xanthine oxidase (XO). It has recently been shown that Trp-containing dipeptides inhibit XO activity (Nongonierma & FitzGerald, 2012).

Quantitative structure activity relationship (QSAR) and molecular docking approaches have

been used to predict peptide binding to proteins (Pripp, 2007). While molecular docking strategies have been widely used as virtual screening tools for development of active substances in the pharmaceutical sector there appears to be limited use of this approach in the study of dietary compounds (Pripp, 2007). Some studies have used docking strategies to identify new peptide sequences with angiotensin converting enzyme (ACE) inhibitory properties or to better understand the interaction of peptides with ACE (Norris, Casey, FitzGerald, Shields, & Mooney, 2012; Pripp, 2007). The peptide docking strategy developed by Norris et al. (2012) led to the discovery of two new dipeptide sequences (Asp-Trp and Trp-Pro) with potent *in vitro* ACE inhibitory properties. A good agreement between the Vina score and IC_{50} value was found for some dipeptides.

No previous docking studies of peptides with XO have been conducted to our knowledge. Molecular docking of various flavonoids with XO has been carried out (Umamaheswari, Madeswaran, Asokkumar, Sivashanmugam, Subhadradevi, & Jagannath, 2012). Various potent DPP-IV inhibitors originating from natural products have recently been identified using a virtual screening procedure. A further increase in the DPP-IV inhibitory properties was achieved using derivatives of the most potent inhibitor (Guasch, et al., 2012). In a recent study, amaranth derived peptides with $\geq$ 13 amino acid residues have been docked with DPP-IV. The mechanism of inhibition of these relatively large peptides has been shown to involve blockage of the active DPP-IV dimer formation (Velarde-Salcedo, Barrera-Pacheco, Lara-González, Montero-Morán, Díaz-Gois, González de Mejia, et al., 2013).

Given the link between diabetes and elevated ROS, it was decided to study the role if amino acids and dipeptides in controlling the activity of DPP-IV and XO. The aim of this study was therefore to determine the potential of amino acids and dipeptides to act as natural XO and DPP-IV inhibitors. Protein-ligand docking (LIGPLOTs) and virtual screening with Autodock Vina were used to assess the XO and DPP-IV inhibitory properties of amino acids and dipeptides. Furthermore, the type of XO and DPP-IV inhibition mediated by the amino acids and dipeptides

was determined using Lineweaver and Burk kinetic analysis.

2. Materials and methods

2.1. Reagents

Porcine DPP-IV (≥ 10 units/mg protein), Gly-Pro-para-nitroaniline (pNA), sodium phosphate monobasic, sodium phosphate dibasic, tris(hydroxymethyl)aminomethane (TRIS), ethylenediamine tretracetic acid (EDTA), hydroxylamine phosphate, xanthine, Allopurinol, bovine XO (0.1-0.4 units/mg protein) were obtained from Sigma Aldrich (Dublin, Ireland). The amino acids and synthetic peptides Trp, Met, Ala, Val, Cys, Leu, His, Ile, Arg, Thr, Glu, Tyr, Asp, Asn, Phe-Leu, His-Leu, Gly-Gln and Ile-Pro-Ile (Diprotin A) were obtained from Sigma-Aldrich while Arg-Trp, Lys-Trp, Trp-Val, Val-Trp, Ile-Trp, Ser-Leu, Ala-Leu, Asp-Lys, Val-Ala, Glu-Lys, Gly-Leu and Ser-Phe were from Bachem (Bubendorf, Switzerland). Pro was from Merck (Darmstadt, Germany), Phe, Glu and Ser were from Sdn BHD (Pandamaran, Klang, Malaysia), Gly was from Fisher Scientific (Dublin, Ireland) and Lys was from SAFC Biosciences (Kansas, USA).

2.2. XO inhibition assay

The test samples (amino acids and dipeptides) were dispersed in HPLC grade water at concentrations ranging from 0.001 to 2.0 $mg.mL^{-1}$. The XO inhibition assay was carried out as essentially described by Nongonierma & FitzGerald (2012). Briefly, test samples (50 µL) were pipetted onto a 96 well microplate (Sarstedt, Dublin, Ireland) containing EDTA (final concentration 12.5 µM), hydroxylamine phosphate (final concentration 25 µM) and xanthine (final concentration 0.125 mM). The reaction was initiated by adding 50 µL of XO (0.1 $U.mL^{-1}$). Each sample was analyzed in quadruplicate. The microplate was incubated at 37°C for 30 min in a microplate reader (Biotek Synergy HT, Winoosky, VT, USA), absorbance of the uric acid formed was monitored at 290 nm. The XO IC_{50} values (concentration of active compound required to observe 50 % inhibition of XO) were determined by plotting the percentage inhibition as a function of the concentration of test compound. The mode of inhibition of the different

compounds was investigated using Lineweaver and Burk kinetic analysis by measuring the initial rate of the reaction at different xanthine concentrations between 0.0125 and 0.1250 mM without inhibitors and in the presence of inhibitors at their IC_{50} concentrations. Km and Vmax values were deducted from the Lineweaver and Burk double reciprocal plots.

2.3. DPP-IV inhibition assay

The test samples were dispersed in HPLC grade water at concentrations ranging from 0.01 to 1.25 $mg.mL^{-1}$. The DPP-IV inhibition assay was carried out as described by Lacroix & Li-Chan (2012a) and Nongonierma and FitzGerald (2013). Test samples (25 µL) were pipetted onto a 96 well microplate containing Gly-Pro-pNA (final concentration 0.200 mM). The negative control contained 100 mM Tris-HCl buffer pH 8.0 (25 µL) and Gly-Pro-pNA. The reaction was initiated by the addition of DPP-IV (final concentration 0.0025 $U.mL^{-1}$). Diprotin A was used as a positive control. Each sample was analysed in triplicate. The microplate was incubated at 37°C for 60 min in a microplate reader, absorbance of the released pNA was monitored at 405 nm. The IC_{50} values for DPP-IV and the mode of inhibition of the different samples were determined as described in Nongonierma and FitzGerald (2013). The initial rate of reaction was measured at different Gly-Pro-pNA concentrations between 0.200 and 0.600 mM without inhibitors and in the presence of inhibitors at their IC_{50} concentrations.

2.4. Computational analysis

AutoDock Vina (Trott & Olsen, 2010) was used to dock all 20 amino acids and 400 dipeptides to Protein Data Bank (PDB) structures 3BDJ, the crystal structure of bovine milk XO with a covalently bound oxipurinol inhibitor (Okamoto, Eger, Nishino, Pai, & Nishino, 2008), 1WCY, the crystal structure of human DPP-IV in complex with Diprotin A (Hiramatsu, Yamamoto, Kyono, Higashiyama, Fukushima, Shima, et al., 2005) and 1ORW, the crystal structure of porcine dipeptidyl peptidase IV (CD26) in complex with a peptidomimetic inhibitor (Engel, Hoffmann, Wagner, Wermann, Heiser, Kiefersauer, et al., 2003). Ten ligand inhibitors of XO were taken from their respective PDB files (Table 1) and docked to the PDB structure 3BDJ. Six ligand inhibitors of

147 DPP-IV were taken from their respective PDB files (Table 1) and docked to the PDB structures
148 1WCY and 1ORW. The initial poses of the PDB-formatted structures of amino acids and
149 dipeptides were generated using the Open Babel Package, version 2.1.1 (O'Boyle, Banck, James,
150 Morley, Vandermeersch, & Hutchison, 2011). AutoDockTools (Morris, Huey, Lindstrom,
151 Sanner, Belew, Goodsell, et al., 2009) was used to prepare the ligands and the two receptors, and
152 to determine the 'search space'. Amino acids, dipeptides and ligand inhibitors were then docked
153 with the PDB structures giving a Vina score i.e., the predicted affinity of the molecule to bind to
154 the PDB structure, calculated in $kcal.mol^{-1}$. A more negative score indicates that a molecule
155 (ligand) is more likely to dock with the structure (enzyme) and achieve more favorable
156 interactions. LIGPLOTs were generated for the highest ranked Vina docking pose for each amino
157 acid, dipeptide and inhibitor (using their PDB files as input and the best Vina docking poses)
158 according to the protocol described by Wallace, Laskowski & Thornton (1995). Dipeptides were
159 also assessed for their intestinal stability using an amino acid clustering model adopted from
160 Foltz, van Buren, Klaffke & Duchateau (2009). This model rates dipeptides as 'stable' (> 75 %
161 peptide remaining after 60 min simulated intestinal digestion- SID) 'neutral' (between 25 and 75
162 % peptide remaining after 60 min SID) or 'unstable' (< 25 % peptide remaining after 60 min
163 SID) with regard to small intestinal stability using correlations between the N- and C-terminal
164 amino acids of dipeptides and the stability of the dipeptide in the intestine. Histograms and scatter
165 plots were generated using the R package (2004).

166 *2.5. Statistical analysis*

167 For experimental data, means comparison was carried out with a one way ANOVA followed
168 by a Tukey's test using SPSS (version 9, SPSS Inc., Chicago, IL, USA) at a significance level $P < 0.05$. For the Lineweaver and Burk plots, experimental data was fitted by linear regression
170 using SPSS.

171 **3. Results**

172 *3.1. XO inhibitory properties of amino acids and dipeptides*

The XO inhibitory properties of 20 amino acids and 15 dipeptides were studied. All six compounds (Trp, Arg-Trp, Trp-Val, Val-Trp, Lys-Trp and Ile-Trp) with XO inhibitory properties contained a Trp residue. Ile-Trp had the lowest IC_{50} value and Lys-Trp the highest IC_{50} value. The IC_{50} value of Ile-Trp was significantly lower ($P < 0.05$) compared to Trp, Trp-Val and Lys-Trp IC_{50} values. In addition, the IC_{50} value of Lys-Trp was significantly higher ($P < 0.05$) compared to that of Arg-Trp and Val-Trp IC_{50} values (Table 2).

The Lineweaver and Burk plots for XO inhibition by Arg-Trp, Lys-Trp and Ile-Trp are shown in Fig. 1. Vmax values were significantly different ($P < 0.05$) with and without inhibitors whereas the Km values were not significantly different ($P \geq 0.05$). This suggests non-competitive inhibition by these dipeptides. Non-competitive XO inhibition by Trp, Val-Trp and Trp-Val has been previously been reported by Nongonierma & FitzGerald (2012).

3.2. DPP-IV inhibitory properties of amino acids and dipeptides

DPP-IV inhibition was observed with 3 amino acids (Met, Leu and Trp). The IC_{50} values for Met, Leu and Trp were 2381.51 ± 25.44, 3419.25 ± 55.68 and 4280.40 ± 48.03 µM, respectively (Table 2). With the exception of Phe-Leu and Trp-Val, the Vina score for Val-Trp (-7.0) was lower than that of the other dipeptides with DPP-IV inhibitory properties (Table 2). However, Val-Trp did not inhibit DPP-IV. Similarly, two other dipeptides with a Vina score ≤ -7.0 (Arg-Trp and Lys-Trp) did not inhibit DPP-IV (Table 2). The Lineweaver and Burk plots for Met, Leu and Trp inhibition of DPP-IV are illustrated in Fig. 2. For Met (Fig. 2a), Leu (Fig. 2b) and Trp (Fig. 2c), the Vmax was not significantly different ($P \geq 0.05$) with and without inhibitors, whereas Km values were significantly different ($P < 0.05$), suggesting a competitive type of inhibition.

3.3. Molecular docking of XO and DPP-IV with amino acids and dipeptides and intestinal stability of dipeptides

The frequency of distribution of the Vina scores for the amino acids, dipeptides and ligand inhibitors is illustrated on Fig. 3 for (a) bovine XO, (b) porcine DPP-IV and (c) human DPP-IV.

For XO, most of the compounds studied had Vina scores between -6.0 and -5.5 (Fig. 3a). By comparison, the Vina scores obtained for quercetin and mercaptopurine, compounds also known to inhibit XO, were -9.5 and -5.7, respectively when docked with 3BJD (Table 1). With the exception of Trp, Phe and Tyr which had values of -6.8, -7.0 and -7.4, respectively, the Vina scores for the amino acids were greater than -6.5 (Table 2). The Vina scores ranged between -4.6 and -7.8 for the 15 dipeptides studied, while all Trp-containing dipeptides had Vina scores ≤ -7.0 (Table 2). Differences in the interaction with XO were evident in the LIGPLOTs for Allopurinol, Trp, Val-Trp and Trp-Val (Fig. 4a, 4b, 4c and 4d). It was seen that docking between XO and Trp or Trp-containing dipeptides in the study herein involved interactions with at least with 6 hydrophobic amino acid residues in XO. On the other hand, only 3 hydrophobic interactions were seen in the docking pose with Allopurinol (Fig. 4a).

For interaction with DPP-IV, most compounds had a score between -7.5 and -6.5 (Fig. 3b and 3c) while the Vina scores for the ligand inhibitors when docked with 1ORW ranged between -8.3 and -5.2 (Table 1). Amino acids had Vina scores between -6.2 and -3.4. Of the 15 dipeptides studied, seven had Vina scores ≤ -6.5, five of which were Trp-containing dipeptides (Table 2). The distribution of Vina scores for the amino acids, dipeptides and ligand inhibitors were similar for porcine and human DPP-IV (data not shown). A linear relationship (slope = 1.03, R^2 = 0.902) was observed between Vina scores for the different compounds studied with 1WCY (human DPP-IV) and 1ORW (porcine DPP-IV) (see supplementary Fig. i). DPP-IV LIGPLOTs for Diprotin A, Trp, Val-Trp and Trp-Val show that hydrophobic interactions and hydrogen bonds may be involved in the binding process (Fig. 4e, 4f, 4g and 4h).

The predicted intestinal stability analysis of the 15 dipeptides was carried out according to Foltz et al. (2009). Five peptides (Ser-Phe, Glu-Lys, Gly-Gln, Asp-Lys and Gly-Leu) were predicted to be stable, one (Trp-Val) neutral and nine (Arg-Trp, Phe-Leu, Lys-Trp, Val-Trp, His-Leu, Ile-Trp, Ala-Leu, Val-Ala and Ser-Leu) unstable (Table 2). Of the 15 dipeptides tested, the dipeptide with the lowest XO and DPP-IV Vina score and predicted to be intestinally stable was

225 Ser-Phe. With the exception of Trp-Val, which was neutral, all Trp-containing dipeptides were
226 predicted to have a low intestinal stability (Table 2).

227 **4. Discussion**

228 Arg-Trp can in theory be released by gastro-intestinal enzyme activities from lactoferrin (LF),
229 Lys-Trp from β-lactoglobulin and Ile-Trp from LF and α-lactalbumin according to *in silico*
230 analysis with the Peptide Cutter Program (ExPaSy, 2011). All other dipeptides with the exception
231 of Phe-Leu, His-Leu, Trp-Val and Gly-Gln, can be released from various milk protein substrates
232 by the action of gastro-intestinal enzyme activities (Nongonierma & FitzGerald, 2012). Trp, Trp-
233 Val and Val-Trp have previously been identified as non-competitive inhibitors of XO. It was
234 shown that the N or C-terminal location of Trp did not affect the ability of Trp-containing
235 dipeptides to inhibit XO as there was no significant difference ($P \geq 0.05$) for the IC_{50} values for
236 both peptides (Nongonierma & FitzGerald, 2012). Three other Trp-containing dipeptides, Ile-Trp,
237 Lys-Trp and Arg-Trp, were shown herein to be non-competitive inhibitors of XO (Table 2, Fig.
238 1). The results herein also show that apart from Trp, none of the other amino acids could inhibit
239 XO (Table 2). The inhibitory properties of Trp have been attributed to their similarity with drug
240 inhibitors of XO which have xanthine-like structures (e.g. Allopurinol) (Nongonierma &
241 FitzGerald, 2012). To our knowledge, no previous studies appear to report on the molecular
242 docking of amino acids and dipeptides to the active site of XO. Molecular docking of ligand
243 inhibitors, amino acids and dipeptides was performed in order to better understand the molecular
244 mechanism of XO inhibition by amino acids and dipeptides. Trp, Tyr and Phe contain C6
245 (phenyl/phenol) structures. However, Tyr and Phe do not inhibit XO, presumably due to the fact
246 that they do not possess xanthine-like structural features. The Vina score for Trp interaction with
247 XO was -6.8, indicative of a good interaction with the active site of XO (Table 2). Surprisingly,
248 the Vina score for Phe and Tyr were -7.0 and -7.4, respectively. The reason(s) for the apparently
249 good fit of those amino acids in the active site requires further investigation. A non-competitive
250 inhibition of XO was observed for Trp and the Trp-containing dipeptides (Fig. 1), suggesting that

these compounds may not interact directly with the active site of XO. The exact nature of the mode of inhibition with Trp and Trp-containing dipeptides requires further studies. While different polyphenolic compounds have also been reported as non-competitive inhibitors of XO their actual mode of inhibition has still not been fully elucidated (Spanou, Veskoukis, Kerasioti, Kontou, Angelis, Aligiannis, et al., 2012).

Most of the peptides with DPP-IV inhibitory properties described in the literature to date contain between two to eight amino acid residues (Lacroix & Li-Chan, 2012b). However, the results herein show that smaller molecular mass components such as amino acids (Trp, Met and Leu) are also able to inhibit DPP-IV (Table 2). The eight dipeptides inhibitors, Trp-Val, Ala-Leu, Glu-Lys, Gly-Leu, Ser-Leu, Phe-Leu, His-Leu and Val-Ala, have previously been identified as DPP-IV inhibitors (Nongonierma & FitzGerald, 2013). Val-Ala has been identified in previous studies as an inhibitor of DPP-IV. This peptide occurs in different milk proteins including α-, β- and κ-casein, β-lactoglobulin and LF (Lacroix & Li-Chan, 2012b). The IC_{50} values found for the dipeptides studied herein are of the same order as the values reported in the literature for other DPP-IV inhibitory peptides. An IC_{50} of 46 μM was reported for Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu (β-casein(f70-77)) while other β-casein derived peptides had IC_{50} values between 110 and 1500 μM (Uenishi, Kabuki, Seto, Serizawa, & Nakajima, 2012). The most potent DPP-IV inhibitory dipeptide herein was Trp-Val (65.69 ± 2.95 μM). However, the reverse peptide Val-Trp did not inhibit DPP-IV (Nongonierma & FitzGerald, 2013). A similar observation has recently been reported in the case of Ile-Pro (IC_{50} 0.41 ± 0.07 μM) where its reverse peptide, Pro-Ile, did not inhibit DPP-IV (Hatanaka, Inoue, Arima, Kumagai, Usuki, Kawakami, et al., 2012). The hydrolytic specificity of DPP-IV shows that the most effective substrates possess a Pro residue in the P_1 position (Engel, Hoffmann, Manhart, Heiser, Chambre, Huber, et al., 2006; Kühn-Wache, Bär, Hoffmann, Wolf, Rahfeld, & Demuth, 2011). Other peptides having Ala, Gly or Ser residues in the P_1 position are also substrates of DPP-IV (Kühn-Wache, Bär, Hoffmann, Wolf, Rahfeld, & Demuth, 2011). The enzymatic mechanism of DPP-IV involves recognition of

277 a charged N-terminus by a Glu-Glu motif and interaction of the amino acid in position P_1 of the
278 substrate with an hydrophobic pocket in the enzyme (Engel, et al., 2006; Guasch, et al., 2012;
279 Kühn-Wache, Bär, Hoffmann, Wolf, Rahfeld, & Demuth, 2011).

280 Different DPP-IV dipeptide inhibitors with a Pro residue at the C terminus have been
281 identified (Hatanaka, et al., 2012). However, other dipeptides which do not contain a Pro residue
282 are also able to inhibit DPP-IV (Lacroix & Li-Chan, 2012b). When Trp was positioned at the C
283 terminus (Val-Trp, Ile-Trp, Arg-Trp, and Lys-Trp), no DPP-IV inhibition was seen in contrast
284 with the dipeptide Trp-Val. Another peptide with a Trp residue at the N terminus, Trp-Pro, has
285 been reported as a DPP-IV inhibitor (Hatanaka, et al., 2012; Lacroix & Li-Chan, 2012b).
286 Inhibition of DDP-IV may correlate with Trp positioned at the N terminus of dipeptides. High
287 DPP-IV inhibition seen with a Trp containing dipeptide has been associated with the fact that the
288 indole group from Trp may interact with the phenyl group of the Ala-Pro-pNA substrate,
289 resulting in a fixation of the substrate to Trp and an apparent higher DPP-IV inhibition (Lorey,
290 Stöckel-Maschek, Faust, Brandt, Stiebitz, Gorrell, et al., 2003). Our data rules out this possibility
291 as no DPP-IV inhibition was seen with the other Trp-containing dipeptides (Val-Trp, Ile-Trp,
292 Arg-Trp, and Lys-Trp). It has been shown that some DPP-IV peptide inhibitors such as Diprotin
293 A (Ile-Pro-Ile) and Diprotin B (Val-Pro-Leu) behave like DPP-IV substrates. The dipeptides Ile-
294 Pro and Val-Pro released by the hydrolytic activity of DPP-IV on Diprotin A and B, respectively,
295 are also inhibitors of DPP-IV (Hatanaka, et al., 2012). It is interesting to note that Ile-Pro, which
296 is released following the hydrolysis of Diprotin A by DPP-IV, has a lower Vina score (-6.6) than
297 that of Diprotin A (-5.9). This may suggest that Ile-Pro is a more potent inhibitor than Diprotin A.
298 Similarly, it has been suggested that some food-derived peptides may present structural analogies
299 with DPP-IV substrates and behave as substrates for this enzyme (Lacroix & Li-Chan, 2012a). In
300 such cases, the peptides may compete with the synthetic substrate used in the DPP-IV assay (Gly-
301 Pro-pNA) for binding to the active site. This would result in a reduction in the release of pNA,
302 leading to a false positive in the DPP-IV assay. However, it is unlikely that amino acids and

303 dipeptides studied herein could act as substrates for DPP-IV as substrates for this enzyme should
304 possess more than 2 amino acid residues (Engel, et al., 2006). Most of the DPP-IV inhibitors
305 studied herein were competitive inhibitors with the exception of Trp-Val which was non-
306 competitive (Table 2). An hydrophobic pocket at the active site of DPP-IV, composed of Tyr-
307 666, Tyr-662, Val-711, Val-656, and Trp-659, has previously been described (Engel, et al.,
308 2003). The LIGPLOTs of Diprotin A and Trp, two competitive inhibitors of DPP-IV, show that
309 these inhibitors interact with the hydrophobic pocket of the active site involving Tyr-666 and
310 Tyr-662 residues of DPP-IV (Fig. 4e and 4f). For the other competitive inhibitors of DPP-IV
311 (Leu, Phe-Leu, His-Leu, Glu-Lys, Ala-Leu, Val-Ala, Ser-Leu and Gly-Leu), the LIGPLOTS
312 (data not shown) revealed hydrophobic interactions with Tyr-666 and also in some cases with
313 Tyr-662 (Phe-Leu, His-Leu, Glu-Lys and Val-Ala). Met was the only inhibitor which could not
314 interact with any of the previously identified amino acid residues of the hydrophobic pocket of
315 DPP-IV. For the other amino acids and dipeptides which were competitive inhibitors of DPP-IV,
316 the Vina scores for DPP-IV did not seem to be in agreement with their experimentally determined
317 IC_{50} value. The Pepsite2 tool (Trabuco, Lise, Petsalaki, & Russell, 2012) was used to determine
318 the site of interaction of Trp-Val with DPP-IV. This analysis indicated a binding site for Trp-Val
319 which was located near the active site of DPP-IV (see supplementary Fig. ii). Trp-Val interacts
320 with DPP-IV near its active site yielding an inhibition which does not affect the affinity of
321 binding (Km) for the substrate. Similar results have been reported for DPP-IV inhibitory peptides
322 derived from the N terminus of the human immunodeficiency virus-1 (HIV) transactivator Tat,
323 which can bind to a secondary site located close to the active site of DPP-IV. Binding of the
324 inhibitors to the secondary site of DPP-IV resulted in a non-competitive inhibition (Lorey, et al.,
325 2003). Docking of peptides ≥ 13 amino acids derived from the tryptic digestion of amaranth has
326 been carried out. The amaranth 11S globulin peptide f(1-13) was shown to interact with the
327 cavity of DPP-IV, thereby blocking access of the substrate. Three peptides > 13 amino acids
328 showed a mode of inhibition which did not involve the active site of DPP-IV. These large

peptides were shown to prevent the formation of the dimeric active form of DPP-IV, resulting in an inhibition of the enzyme (Velarde-Salcedo, et al., 2013). Interestingly, Trp and Trp-Val present a dual bioactivity, inhibiting both DPP-IV and XO. Trp-Val was shown to be a non-competitive inhibitor of XO and DPP-IV, suggesting a binding outside the active site of the enzyme. In this instance, docking of Trp-Val to the active site cannot be utilized to determine interaction of the peptides with XO or DPP-IV. More knowledge is needed in terms of the structure of the secondary binding site of XO and DPP-IV in order to dock the non-competitive inhibitors to this site.

Prediction of the intestinal stability indicates that most dipeptides were unstable (Table 2). It has been noted by Foltz et al. (2009) that the low intestinal stability of Trp-containing dipeptides could be detrimental to their bioactivity (ACE inhibitory properties). However Trp, and the Trp-containing dipeptides (Arg-Trp, Trp-Val, Val-Trp, Lys-Trp and Ile-Trp) were able to inhibit XO. Therefore, it could be hypothesised that bioactivity may be retained even following hydrolysis of the dipeptides Arg-Trp, Trp-Val, Val-Trp, Lys-Trp and Ile-Trp by intestinal peptidases. Simulated intestinal digestion of a gastric LF hydrolysate was performed and the XO inhibitory properties of the subsequent hydrolysate were not impaired. This suggests that Trp and Trp-containing components from this LF hydrolysate were still bioactive (Nongonierma & FitzGerald, 2012). It has been shown that LF hydrolysates which contain Trp and Trp-containing dipeptides could inhibit XO. A consequence of Trp-containing dipeptide cleavage by intestinal enzymes may be reduced bioavailability of Trp compared to the dipeptide. It has been shown that dipeptide uptake *in vivo* is more efficient than that for free amino acids (Adibi & Morse, 1971). In order to interact with XO, an inhibitor should cross the gut barrier and survive serum peptidase activity in order to reach locations containing XO (i.e. plasma, liver, gut, lung, kidney, brain and heart). However as hydrophobic peptides have been associated with a high intestinal trans-epithelial permeation, Trp and Trp-containing dipeptides may be good candidates for XO inhibition as suggested elsewhere (Nongonierma & FitzGerald, 2012).

In the case of DPP-IV, Trp-Val and Trp were also inhibitors. However, the IC_{50} of Trp was more than 60 times higher than that of Trp-Val, suggesting that hydrolysis of Trp-Val would result in a significant reduction in the overall DPP-IV inhibitory properties. The simulated gastrointestinal digestion (SGID) of DPP-IV inhibitory peptides from rice bran has been studied (Huang, Jao, Ho, & Hsu, 2012). Different effects of SGID on the bioactivity of the peptides were seen, showing no effect or an increase in the DPP-IV inhibition depending on the peptide sequence (Huang, Jao, Ho, & Hsu, 2012). In another study, SGID of amaranth glutelin resulted in an increase in DPP-IV inhibitory properties compared to raw amaranth flour (Velarde-Salcedo, et al., 2013). The fact that some amino acids behave as DPP-IV inhibitors suggests that release of these amino acid residues following cleavage of the dipeptides may affect the overall inhibitory activity. For example, not all Trp-containing dipeptides (i.e. Arg-Trp, Lys-Trp, Val-Trp and Ile-Trp) inhibited DPP-IV in contrast with Trp. Intestinal instability of these dipeptides could increase DPP-IV inhibitory activity following the release of Trp. In the case of Leu-containing dipeptides (i.e. Phe-Leu, Ala-Leu, Ser-Leu and Gly-Leu), cleavage of the dipeptides will result in a decrease in the potency of the sample as the IC_{50} value for Leu is between 1.3 and 23.9 times greater than that of the dipeptides. Based on the absence of DPP-IV inhibition with Val and Ala, it is predicted that cleavage of Val-Ala, which is intestinally unstable, would result in a loss in DPP-IV inhibitory activity.

A natural avenue for the prevention of type 2 diabetes may involve the incorporation of peptides in the diet of prediabetics or type 2 diabetic subjects. This study has demonstrated the possibility of targeting inhibition of XO and DPP-IV with amino acids and dipeptides as a means to reduce the generation of ROS and to enhance the half-life of incretins. Multi-functional foods containing amino acids and bioactive peptides may have potential in targeting different bioactivities. Further evaluation of Trp-Val is needed to verify its bioavailability and potential health benefits *in vivo*.

Conclusion

Due to the non-competitive XO and DPP-IV inhibition seen with Trp-Val, there appears to be some limitations in the use of Vina scores determined by docking to the active sites of XO and DPP-IV. There is a lack of detailed information in the literature in terms of XO and DPP-IV inhibition by amino acids and dipeptides. Generation of additional experimental data for XO and DPP-IV inhibition with various dipeptides would be useful to validate bioinformatics based models for the discovery of new dipeptides sequences which act as inhibitors of these enzymes. This may lead to the identification of food-derived bioactive peptides which may be formulated into functional foods.

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Table 1 Summary details of the protein database bank (PDB) structures and associated Vina scores of xanthine oxidase (XO) and dipeptidyl peptidase IV (DPP-IV) complexed with different ligands.

Protein database identification code	Description	Vina score*
	Xanthine oxidase (XO)	
3BDJ	Crystal structure of bovine milk xanthine dehydrogenase with a covalently bound oxipurinol inhibitor	-7.5
1N5X	Xanthine dehydrogenase from bovine milk with inhibitor TEI-6720 bound	-8.2
1V97	Crystal structure of bovine milk xanthine dehydrogenase FYX-051 bound form	-8.5
3AM9	Complex of bovine xanthine dehydrogenase and trihydroxy FYX-051	-9.1
3ETR	Crystal structure of xanthine oxidase in complex with lumazine	-7.7
3NRZ	Crystal structure of bovine xanthine oxidase in complex with hypoxanthine	-6.7
3NS1	Crystal structure of bovine xanthine oxidase in complex with 6-mercaptopurine	-5.7
3NVW	Crystal structure of bovine xanthine oxidase in complex with guanine	-7.1
3NVY	Crystal structure of bovine xanthine oxidase in complex with quercetin	-9.5
3NVZ	Crystal structure of bovine xanthine oxidase in complex with indole-3-aldehyde	-7.7
	Dipeptidyl peptidase (DPP-IV)	
1ORW	Crystal structure of porcine dipeptidyl peptidase IV (CD26) in complex with a peptidomimetic inhibitor	-5.9
1WCY	Crystal structure of human dipeptidyl peptidase IV (DPPIV) complex with Diprotin A	-6.2
2AJ8	Porcine dipeptidyl peptidase IV (CD26) in complex with 7-benzyl-1,3-dimethyl-8-piperazin-1-yl-3,7-dihydro-purine-2,6-dione (BDPX)	-7.8
2AJC	Porcine dipeptidyl peptidase IV (CD26) in complex with 4-(2-aminoethyl)-benzene sulphonyl fluoride (AEBSF)	-5.2
2BUA	Crystal structure of porcine dipeptidyl peptidase IV (CD26) in complex with a low molecular weight inhibitor	-5.6
2BUC	Crystal structure of porcine dipeptidyl peptidase IV (CD26) in complex with a tetrahydroisoquinoline inhibitor	-8.3

*Vina scores of the ligand provided with the structure back to the structure of bovine XO with a covalently bound oxipurinol inhibitor (3BJD) and porcine DPP-IV with a peptidomimetic inhibitor (1ORW).

Table 2 Summary of the Vina score, test compound concentration inducing 50 % inhibition (IC_{50}) of xanthine oxidase (XO) and dipeptidyl peptidase IV (DPP-IV) along with inhibition type for the dipeptides and amino acids with predicted intestinal stability of the dipeptides. IC_{50} values are mean ± confidence interval.

Compound type	Sequence	Predicted stability[#]	Xanthine oxidase			Dipeptidyl peptidase IV		
			Vina score 3BDJ[†]	IC_{50} XO bovine(μM)[‡]	Type of inhibition[*]	Vina score 1ORW[§]	IC_{50} DPP-IV porcine (μM)[##]	Type of inhibition[**]
Dipeptide	RW	unstable	-7.8	1136.9 ± 94.7[b,c]	non-competitive	-7.5	-	-
	FL	unstable	-7.7	-	-	-7.6	399.58 ± 10.81[d]	competitive
	KW	unstable	-7.2	1450.9 ± 183.0[d]	non-competitive	-7.2	-	-
	WV	neutral	-7.2	1301.7 ± 336.7[c,d]	non-competitive	-8.1	65.69 ± 2.95[b]	non-competitive
	VW	unstable	-7.0	1195.7 ± 131.5[b,c]	non-competitive	-7.6	-	-
	SF	stable	-6.9	-	-	-7.0	-	-
	HL	unstable	-6.5	-	-	-6.7	143.19 ± 0.35[c]	competitive
	IW	unstable	-6.2	1046.6 ± 67.0[b]	non-competitive	-7.7	-	-
	EK	stable	-5.9	-	-	-6.3	3216.75 ± 2.12[h]	competitive
	GQ	stable	-5.6	-	-	-5.8	-	-
	DK	stable	-5.4	-	-	-6.4	-	-
	AL	unstable	-4.9	-	-	-5.7	882.13 ± 68.66[e]	competitive
	VA	unstable	-5.7	-	-	-5.4	168.24 ± 7.96[c]	competitive
	SL	unstable	-4.8	-	-	-5.7	2517.08 ± 36.33[f]	competitive
	GL	stable	-4.6	-	-	-5.7	2615.03 ± 612.80[g]	competitive
Amino acid	W	na	-6.8	1259.8 ± 84.5[c,d]	non-competitive	-6.2	4280.40 ± 48.03[h]	competitive
	M	na	-4.1	-	-	-4.0	2381.51 ± 25.44[f]	competitive
	L	na	-5.0	-	-	-4.3	3419.25 ± 55.68[h]	competitive
	V	na	-5.3	-	-	-4.2	-	-
	A	na	-4.6	-	-	-3.7	-	-
	C	na	-4.5	-	-	-3.7	-	-
	D	na	-5.0	-	-	-4.8	-	-
	E	na	-5.6	-	-	-5.1	-	-
	F	na	-7.0	-	-	-5.5	-	-
	G	na	-3.9	-	-	-3.4	-	-
	H	na	-5.3	-	-	-4.8	-	-
	I	na	-4.1	-	-	-4.7	-	-

	K	na	-6.0	-	-	-4.4	-	-
	N	na	-5.0	-	-	-4.6	-	-
	P	na	-4.2	-	-	-4.5	-	-
	Q	na	-5.4	-	-	-5.3	-	-
	R	na	-5.8	-	-	-4.7	-	-
	S	na	-4.8	-	-	-3.9	-	-
	T	na	-5.1	-	-	-4.8	-	-
	Y	na	-7.4	-	-	-5.7	-	-
Inhibitor	Allopurinol	na	-7.3	5.5 ± 0.1^{a}	competitive	na	na	na
	IPI	na	na	na	na	-5.9	4.23 ± 0.08^{a}	competitive

na: not applicable

[#]Predicted stability was determined according to Foltz et al. (2009).

[†]Crystal structure of bovine milk xanthine dehydrogenase with a covalently bound oxipurinol inhibitor

[‡] Values represent mean IC_{50} values ± confidence interval (*P = 0.05*) for quadruplicate determination (n=4). Values with different superscript letters are significantly different (*P < 0.05*). For XO, IC_{50} values of W, VW and WV were taken from Nongonierma & FitzGerald (2012).

*Type of inhibition determined using Lineweaver and Burk plots as described in Nongonierma & FitzGerald (2012)

[§]Crystal structure of porcine DPP-IV in complex with a peptidomimetic inhibitor

**Type of inhibition determined using Lineweaver and Burk plots as described in Nongonierma & FitzGerald (2013)

[##]Values represent mean IC_{50} values ± confidence interval (*P = 0.05*) for triplicate determination (n=3). Values with different superscript letters are significantly different (*P < 0.05*). IC_{50} values for the dipeptides were taken from Nongonierma & FitzGerald (2013)

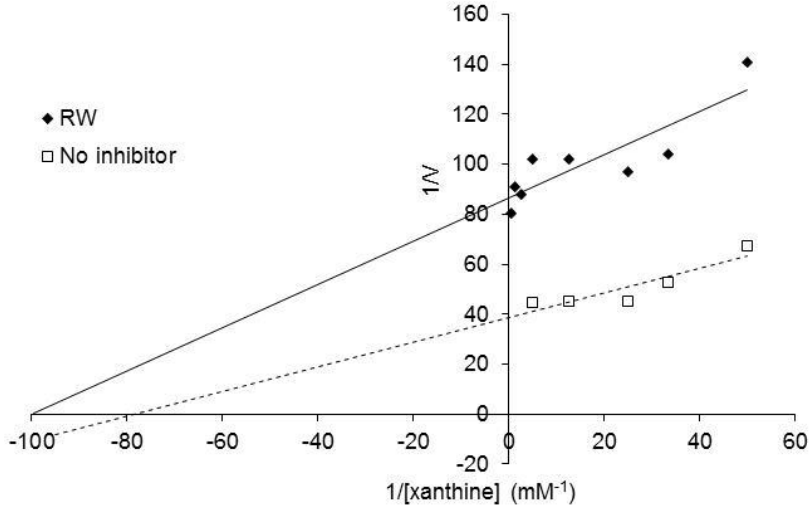
Figure captions

Fig. 1. Lineweaver and Burk plots for xanthine oxidase (XO) inhibition with Arg-Trp (a), Lys-Trp (b) and Ile-Trp (c). Each data point is the average of 6 values.

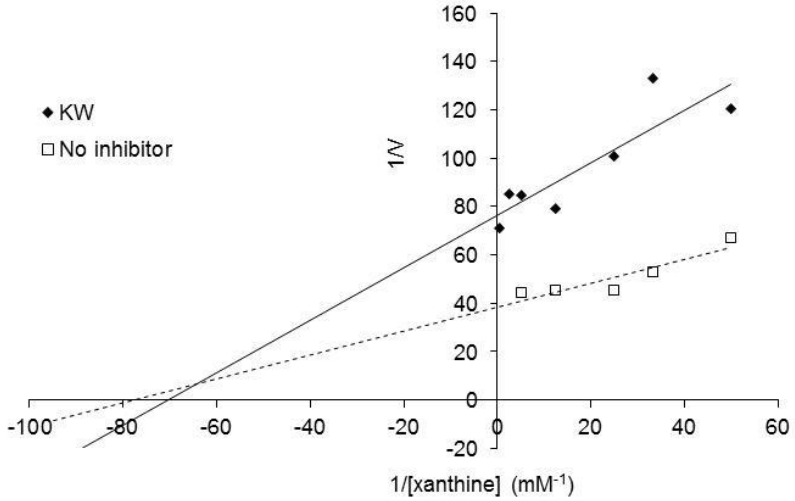
Fig. 2. Lineweaver and Burk plots for dipeptidyl peptidase IV (DPP-IV) with Met (a), Leu (b) and Trp (c). Each data point is the average of 6 values.

Fig. 3. Frequency of distribution of Vina scores for predicted binding of the 20 amino-acids, 400 possible dipeptides and ligands to xanthine oxidase (XO) and dipeptidyl peptidase IV (DDP-IV). Bovine XO (a), porcine DDP-IV (b) and human DDP-IV (c).

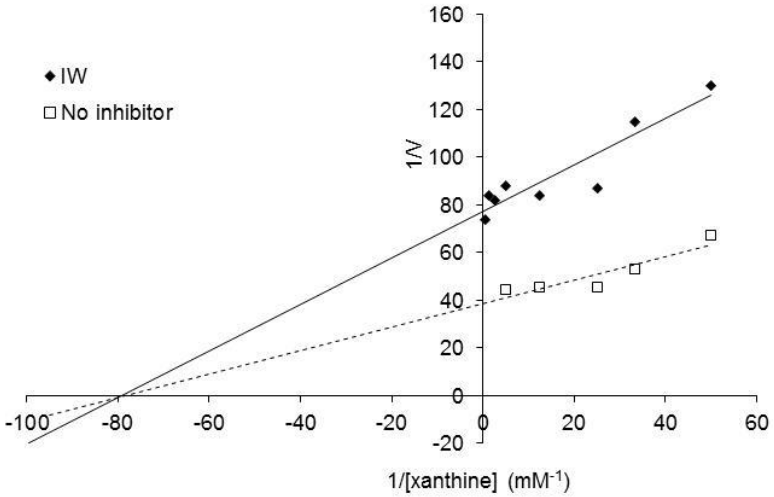
Fig. 4. LIGPLOT profiles of the crystal structure of bovine milk xanthine oxidase (XO) with Allopurinol inhibitor (a), Trp (b), Val-Trp (c) and Trp-Val (d) and LIGPLOTs of the crystal structure of human dipeptidyl peptidase IV (DPP-IV) complex with Diprotin A (e), Trp (f), Val-Trp (g) and Trp-Val (h)


RW
No inhibitor
1/V
1/[xanthine] (mM-1)

(a)


KW
No inhibitor
1/V
1/[xanthine] (mM-1)

(b)

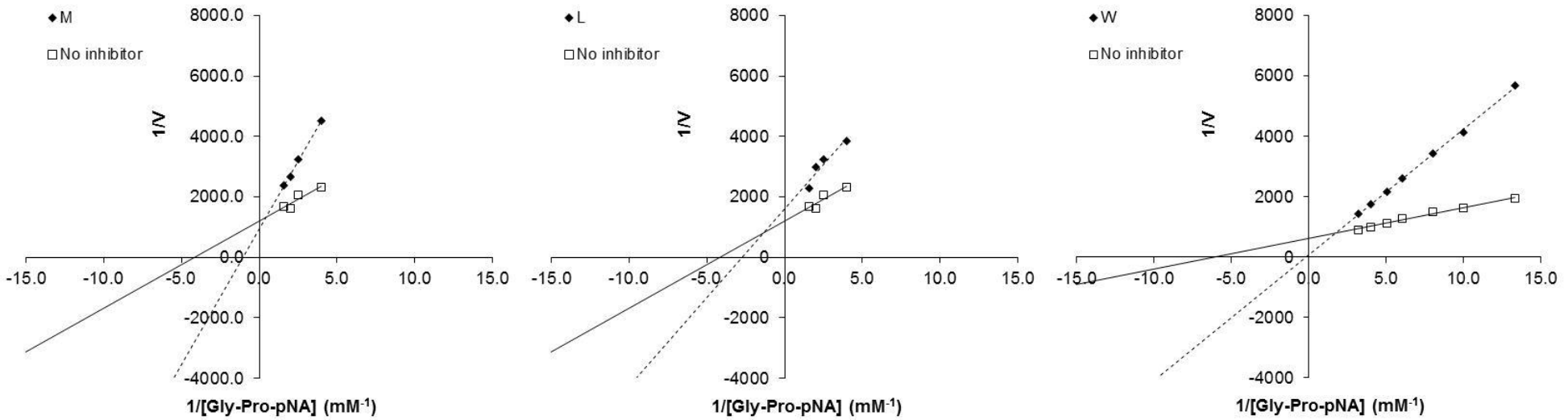

IW
No inhibitor
1/V
1/[xanthine] (mM-1)

(c)

Fig. 1

566

(a) (b) (c)



567

568 **Fig. 2**

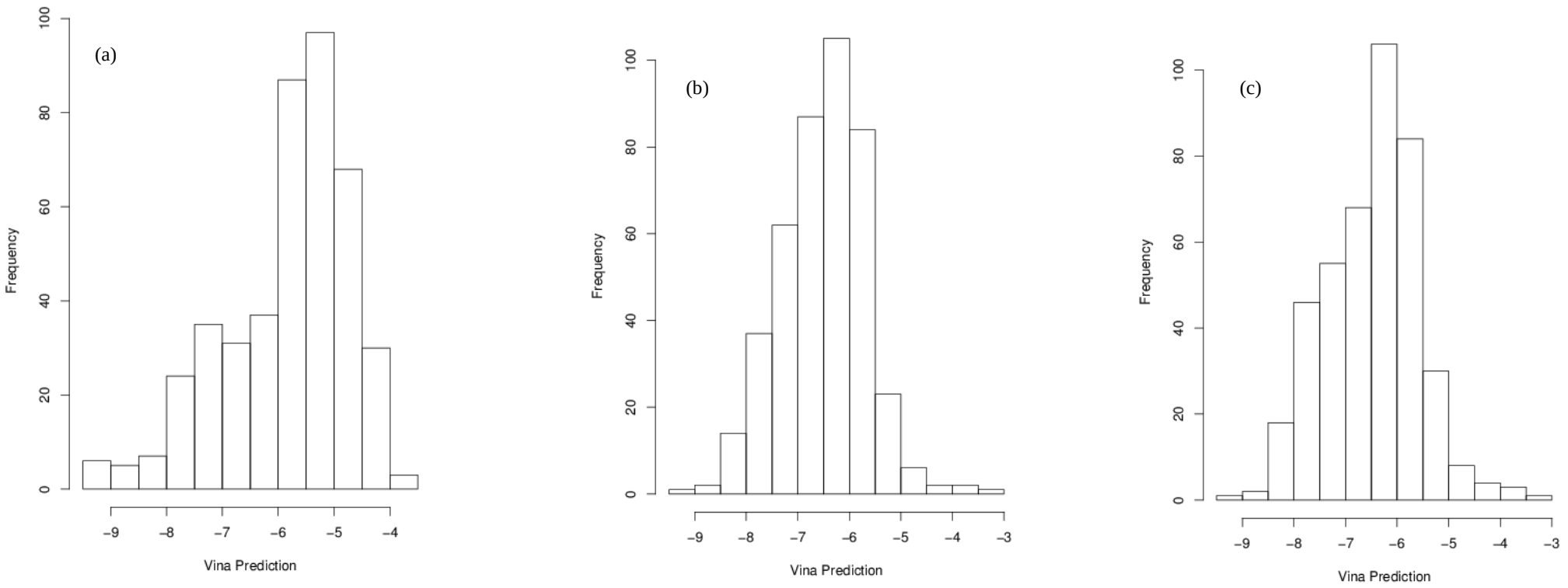
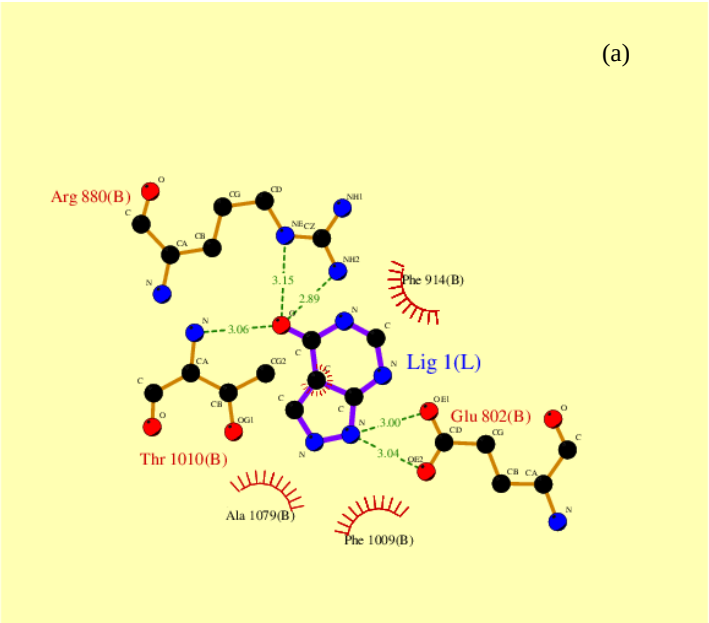
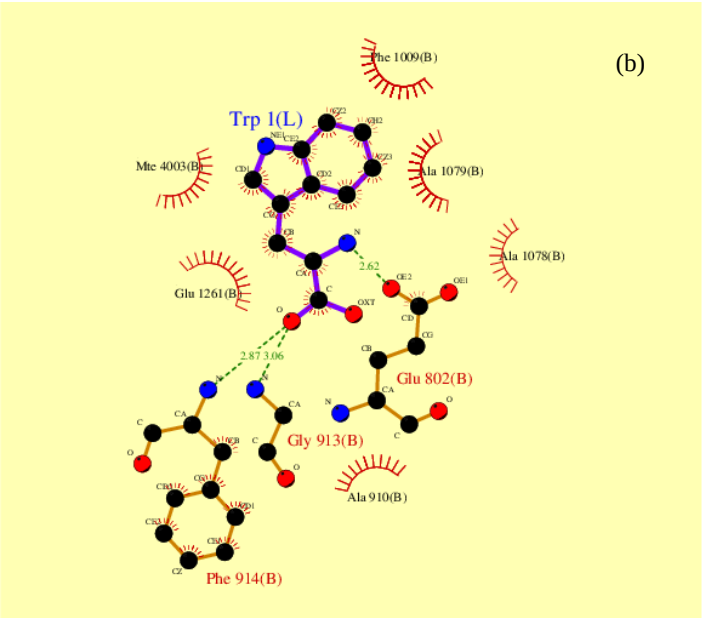
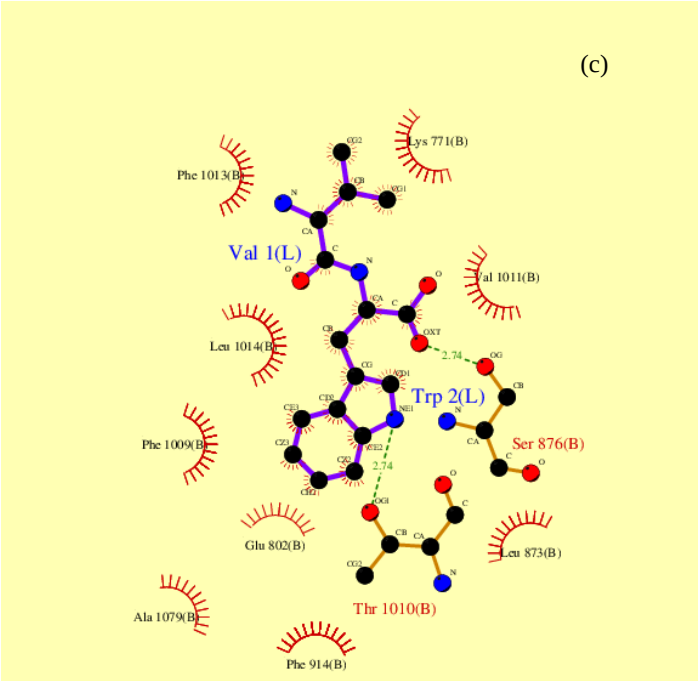
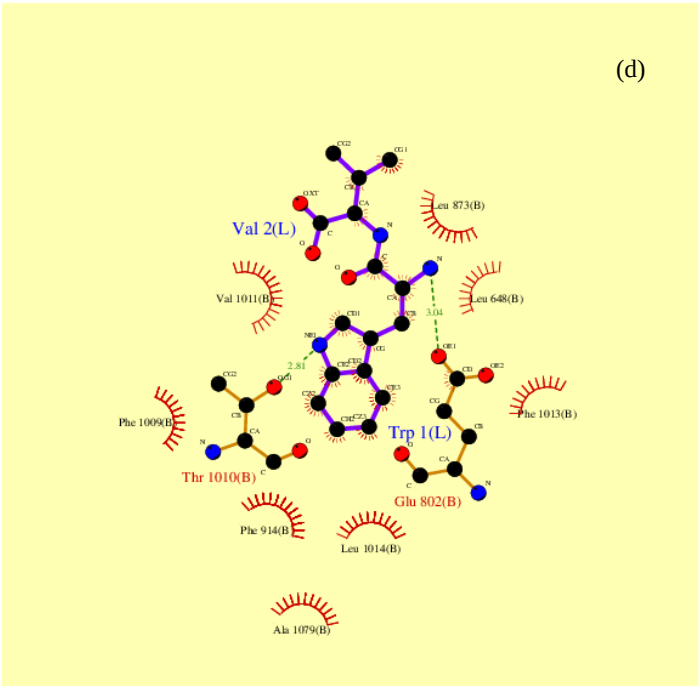

(a)
Frequency
Vina Prediction
(b)
Frequency
Vina Prediction
(c)
Frequency
Vina Prediction

Fig. 3

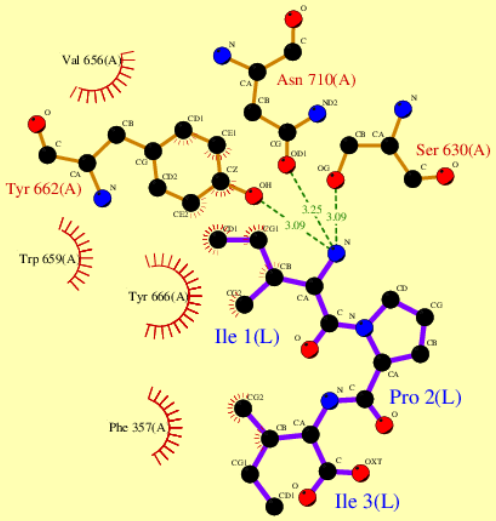

(a)
Arg 880(B)
Phe 914(B)
Lig 1(L)
Glu 802(B)
Thr 1010(B)
Ala 1079(B)
Phe 1009(B)


(b)
Phe 1009(B)
Trp 1(L)
Mte 4003(B)
Ala 1079(B)
Ala 1078(B)
Glu 1261(B)
Glu 802(B)
Gly 913(B)
Ala 910(B)
Phe 914(B)

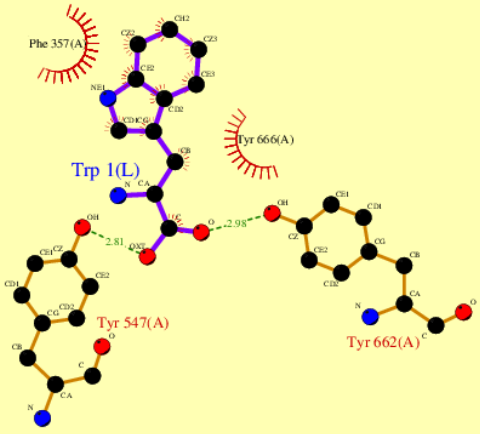

(c)
Lys 771(B)
Phe 1013(B)
Val 1(L)
Val 1011(B)
Leu 1014(B)
Trp 2(L)
Phe 1009(B)
Ser 876(B)
Glu 802(B)
Leu 873(B)
Ala 1079(B)
Thr 1010(B)
Phe 914(B)


(d)
Leu 873(B)
Val 2(L)
Val 1011(B)
Leu 648(B)
Phe 1009(B)
Trp 1(L)
Phe 1013(B)
Thr 1010(B)
Glu 802(B)
Phe 914(B)
Leu 1014(B)
Ala 1079(B)

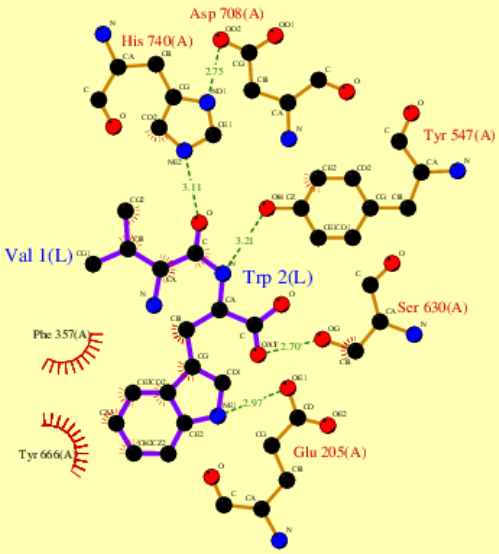
(e)


Val 656(A)
Asn 710(A)
Ser 630(A)
Tyr 662(A)
Trp 659(A)
Tyr 666(A)
Ile 1(L)
Pro 2(L)
Phe 357(A)
Ile 3(L)

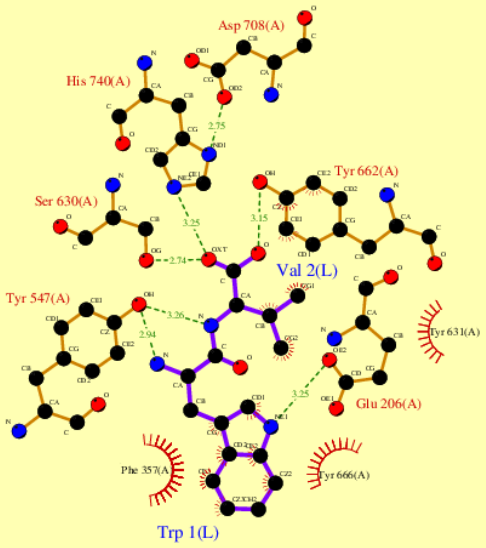
(f)

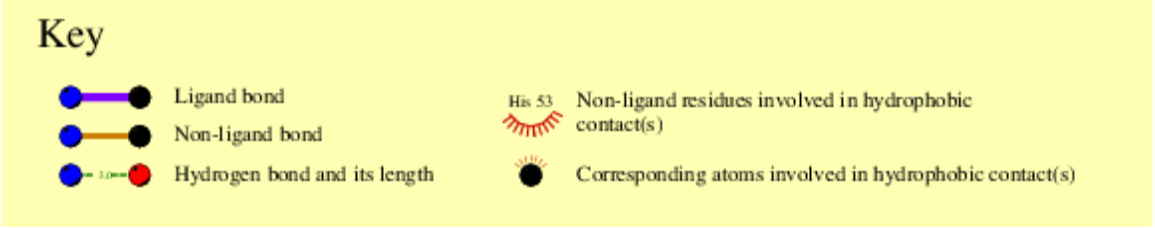

Phe 357(A)
Tyr 666(A)
Trp 1(L)
Tyr 547(A)
Tyr 662(A)

(g)


Asp 708(A)
His 740(A)
Tyr 547(A)
Val 1(L)
Trp 2(L)
Ser 630(A)
Phe 357(A)
Tyr 666(A)
Glu 205(A)

(h)


Asp 708(A)
His 740(A)
Tyr 662(A)
Ser 630(A)
Val 2(L)
Tyr 547(A)
Tyr 631(A)
Glu 206(A)
Phe 357(A)
Tyr 666(A)
Trp 1(L)


Key
Ligand bond
Non-ligand bond
Hydrogen bond and its length
His 53
Non-ligand residues involved in hydrophobic contact(s)
Corresponding atoms involved in hydrophobic contact(s)

615

616 **Fig. 4**

Supplementary data

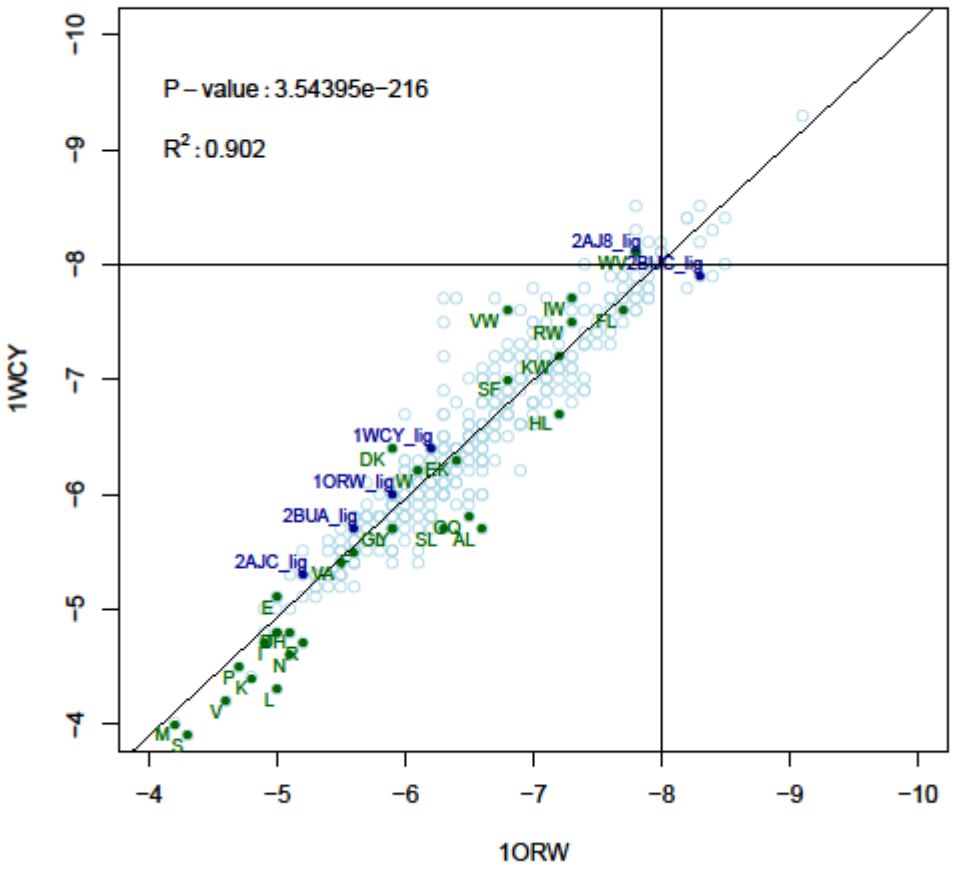


Fig. i Correlation between the prediction of binding of the 20 amino-acids, 400 possible dipeptides to dipeptidyl peptidase IV (DPP-IV) binding site from porcine (1ORW) and human origin (1CWY).

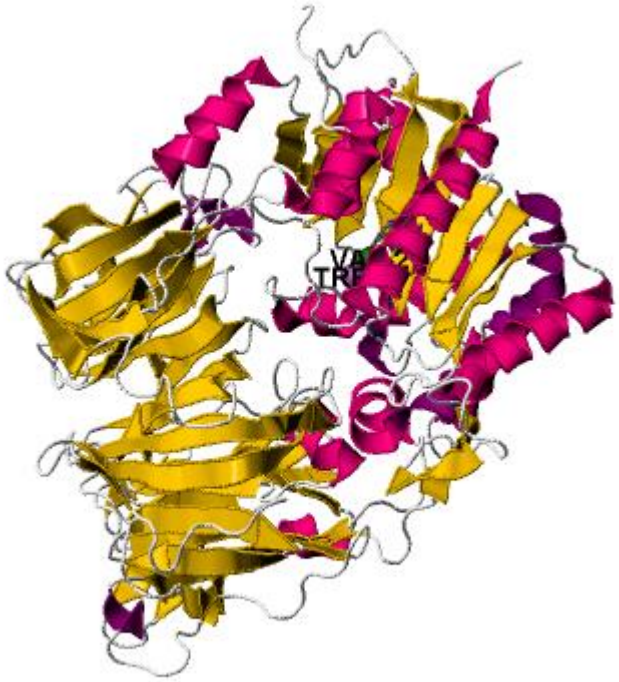


Fig. ii Dipeptidyl peptidase IV (DPP-IV) 1WCY structure with potential location for the dipeptide Trp-Val close to the active site, as defined by Pepsite2 (Trabuco, Lise, Petsalaki, & Russell, 2012) (*P=0.0037*) suggesting that binding is most likely to occur near the active site.