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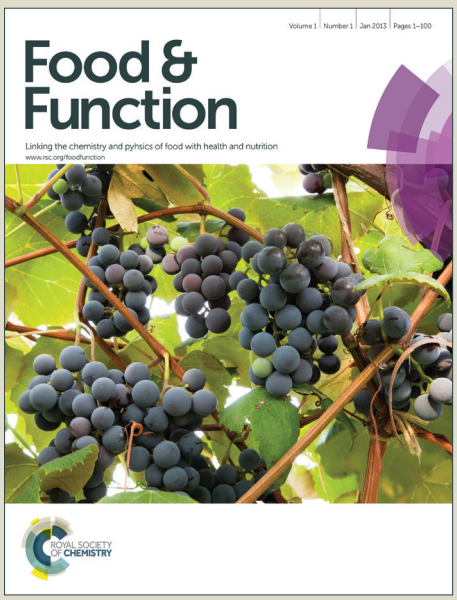

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Utilisation of the isobole methodology to study dietary peptide-drug and peptide-peptide interactive effects on dipeptidyl peptidase IV (DPP-IV) inhibition

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Inhibition of dipeptidyl peptidase-IV (DPP-IV) is used as a means to regulate post-prandial serum glucose in type 2 diabetics. The effect of drug (Sitagliptin®)/peptide and binary peptide mixtures on DPP-IV inhibition was studied using an isobole approach. Five peptides (Ile-Pro-Ile-Gln-Tyr, Trp-Lys, Trp-Pro, Trp-Arg and Trp-Leu), having DPP-IV half maximum inhibitory concentration values (IC_{50}) < 60 µM and reported to act through different inhibition mechanisms, were investigated. The dose response relationship of Sitagliptin/peptide (1:0, 0:1, 1:852, 1:426 and 1:1704 on a molar basis) and binary Ile-Pro-Ile-Gln-Tyr/peptide (1:0, 0:1, 1:1, 1:2 and 2:1 on a molar basis) mixtures for DPP-IV inhibition was characterised. Isobolographic analysis showed, in most instances, an additive effect on DPP-IV inhibition. However, a synergistic effect was observed with two Sitagliptin: Ile-Pro-Ile-Gln-Tyr (1:426 and 1:852) mixtures and an antagonistic effect was seen with one Sitagliptin:Trp-Pro (1:852) mixture, and three binary peptide mixtures (Ile-Pro-Ile-Gln-Tyr:Trp-Lys (1:1 and 2:1) and Ile-Pro-Ile-Gln-Tyr:Trp-Leu (1:2)). The results show that Sitagliptin and food protein-derived peptides can interact, thereby enhancing overall DPP-IV inhibition. Combination of Sitagliptin with food protein-derived peptides may help in reducing drug dosage and possible associated side-effects.

Key words

dipeptidyl peptidase IV inhibitors, type 2 diabetes, bioactive peptides, Sitagliptin, isobole methodology, dietary peptide-drug interactions

1. Introduction

The increasing global prevalence of type 2 diabetes (T2D) has led the scientific community to investigate different strategies in order to slow down its evolution. Dipeptidyl peptidase IV (DPP-IV) inhibitors belong to a new class of drugs with an antidiabetic action, with Sitagliptin® (Januvia®, Merck & Co., Inc. USA) being the first DPP-IV inhibitor launched on the market. DPP-IV cleaves incretins such as glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) *in vivo*. Inhibition of DPP-IV therefore increases the half-life of incretins, thereby promoting insulin secretion from pancreatic beta cells [1].

Food protein-derived bioactive peptides have been shown to positively affect biomarkers of T2D such as postprandial glycaemia and insulin secretion [2-4]. It is thought that the antidiabetic properties of specific food protein hydrolysates may arise from their DPP-IV inhibitory activity [5,6]. Food protein hydrolysates, originating mostly from milk, have been reported for their DPP-IV inhibitory potential [7]. The peptides therein may inhibit DPP-IV through different modes of inhibition [8-10]. In a physiological situation, it is expected that different food protein-derived peptides may concomitantly inhibit DPP-IV. However, the contribution of multiple food protein-derived peptides, as present in food protein hydrolysates, to overall DPP-IV inhibition has not been determined. The combination of milk protein-derived peptides with Sitagliptin was recently shown to have an additive effect on DPP-IV inhibition [11]. However, to date the interactive effects of peptide-peptide and peptide-drug combinations on DPP-IV inhibition does not appear to have been extensively studied.

The interactive effects of drug mixtures is conventionally studied using an isobole methodology [12,13]. It has been recently proposed that using combinations of antidiabetic drugs and phytochemicals may be a new approach to help reduce the side-effects observed during drug intake [13]. Synergistic antidiabetic activity has been shown *in vivo* when combinations of phytochemicals (ferulic acid) and antidiabetic drugs (metformin and thiazolidinedione) were employed [14]. To our understanding, the isobole method has not been previously applied to determine interactive effects between drug/peptide or binary peptide mixtures. The aim of this study was therefore to utilise an isobole methodology to study the interactions between Sitagliptin and food protein-derived DPP-IV inhibitory peptides, and between binary mixtures of DPP-IV inhibitory peptides.

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Table 1: Summary of the peptide cutter analysis using gastrointestinal enzyme activities to release Trp-Lys, Trp-Arg, Trp-Leu and Ile-Pro-Ile-Gln-Tyr from different food proteins.

Peptide†	Protein fragment	Enzyme	Protein source	Protein
Trp-Lys	40-41	pepsin	Oat (*Avena sativa*)	Avenin
Trp-Arg	212-213	trypsin	Wheat (*Triticum aestivum*)	Large subunit RuBisCO*
Trp-Arg	212-213	trypsin	Barley (*Hordeum vulgare*)	Large subunit RuBisCO
Trp-Arg	212-213	trypsin	Oat (*Avena sativa*)	Large subunit RuBisCO
Trp-Arg	212-213	trypsin	Corn (*Zea mays*)	Large subunit RuBisCO
Trp-Arg	212-213	trypsin	Rice (*Oryza sativa subsp. Japonica*)	Large subunit RuBisCO
Trp-Arg	212-213	trypsin	Sorghum (*Sorghum vulgare*)	Large subunit RuBisCO
Trp-Arg	171-172	trypsin	Quinoa (*Chenopodium quinoa*)	RuBisCO large chain
Trp-Arg	212-213	trypsin	Amaranth (*Amaranthus hypochondriacus*)	Large subunit RuBisCO
Trp-Arg	207-208	trypsin	*Palamaria palmata* (*Rhodymenia palmata*)	Allophycocyanin α chain
Trp-Arg	171-172	trypsin	*Palamaria palma*ta (*Rhodymenia palmata*)	Allophycocyanin β chain
Trp-Arg	212-213	trypsin	*Palamaria palmata* (*Rhodymenia palmata*)	Phycocyanin α
Trp-Leu	104-105	elastase	Bovine milk (*Bos taurus*)	α-lactalbumin
Ile-Pro-Ile-Gln-Tyr	26-30	chymotrypsin	Bovine milk (*Bos taurus*)	κ-casein

†Peptide sequence using the three letter code

*RuBisCO: Ribulose bisphosphate carboxylase

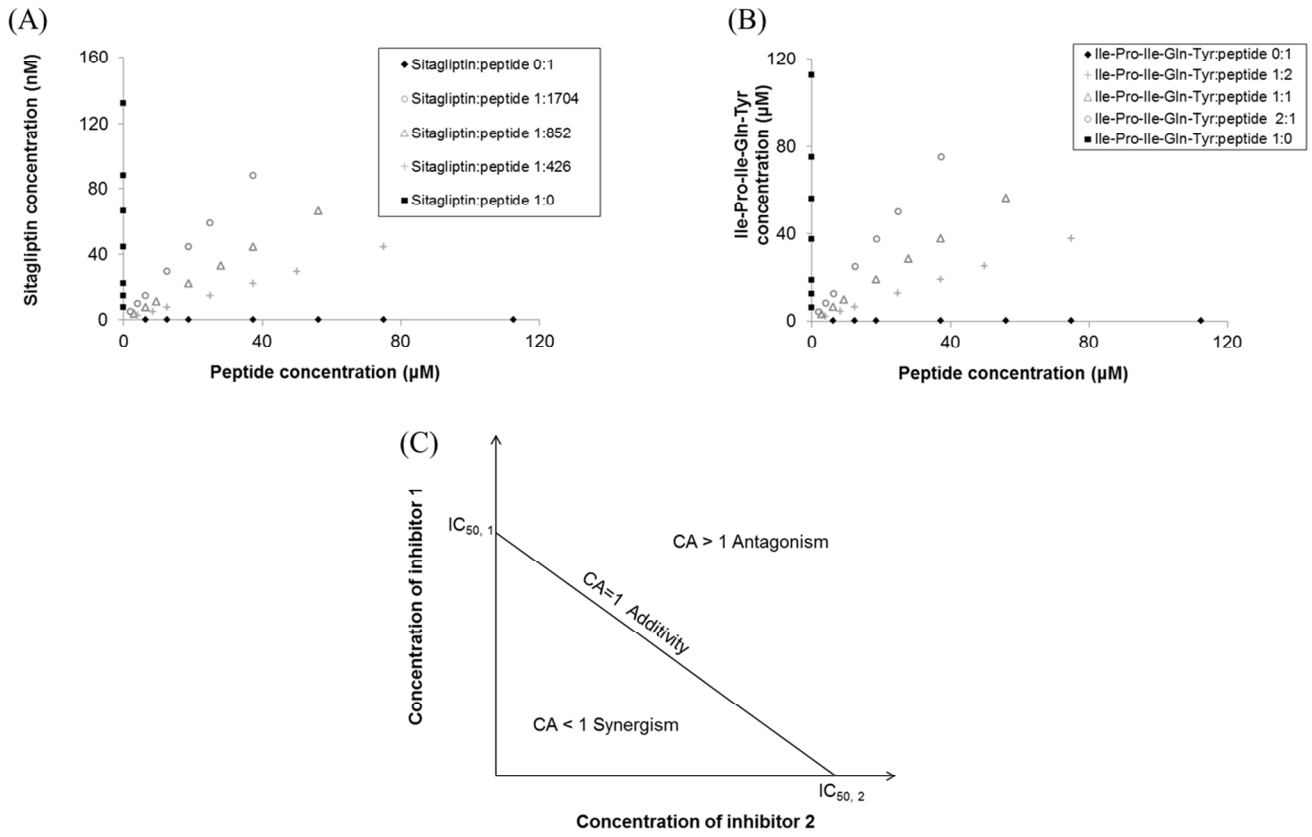

Fig 1: Experimental design used to study the dose response effect of (A) different Sitagliptin/peptide (Ile-Pro-Ile-Gln-Tyr, Trp-Lys, Trp-Pro, Trp-Arg and Trp-Leu) and (B) Ile-Pro-Ile-Gln-Tyr:peptide (Trp-Lys, Trp-Pro, Trp-Arg and Trp-Leu) mixtures on dipeptidyl peptidase IV (DPP-IV) inhibition. (C) Schematic representation of a 50% inhibition isobole diagram and interpretation of the type of interactions between two inhibitors based on the concentration addition (CA) value of the mixture. IC_{50}: half maximum inhibitory concentration.

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2. Material and methods

2.1. Reagents

Porcine DPP-IV (≥ 10 Units mg^{-1} protein), Gly-Pro-pNA, tris(hydroxymethyl)aminomethane (TRIS), Ile-Pro-Ile and Sitagliptin were from Sigma Aldrich (Dublin, Ireland). Trp-Pro, Trp-Arg and Ile-Pro-Ile-Gln-Tyr were obtained from Thermo Fisher Scientific (Ulm, Germany) while Trp-Leu and Trp-Lys were from Bachem (Bubendorf, Switzerland). Hydrochloric acid (HCl) and high-performance liquid chromatography (HPLC) grade water were from VWR (Dublin, Ireland).

2.2. *In silico* analysis of food proteins

The occurrence of the five DPP-IV inhibitory peptides used in this study was determined *in silico* in 72 dietary proteins [15] (Supplementary Table S1). The sequence of the mature protein (without the propeptide) were obtained from UniProt using the ExPASy resource portal. The occurrence of the peptides was determined using an in-house generated Matlab programme (version R2014b, MathWorks, Inc, Natick, MA, USA). Proteins with the five peptides were further subjected to *in silico* digestion with gastrointestinal enzymes (pepsin, trypsin, chymotrypsin and elastase) using the Peptide Cutter facility in Matlab.

2.3. Experimental design to study Sitagliptin-peptide and peptide-peptide interactions

Stock solutions of peptides (900 µM) and Sitagliptin (1056 nM) were prepared to yield ~ 80 % DPP-IV inhibition. The ratios studied for the binary peptide mixtures were as described by Tallarida [16]. The same volumetric mixtures of peptide stock solutions (i.e. 1:1, 1:2 and 2:1) were also employed for the Sitagliptin:peptide mixtures. For the binary peptide mixtures, only the combinations with the most potent substrate-type competitive DPP-IV inhibitor, Ile-Pro-Ile-Gln-Tyr (IC_{50} value of 23 µM), and non-competitive (Trp-Lys, Trp-Pro and Trp-Arg) and competitive (Trp-Leu) DPP-IV inhibitors were studied. The mixtures consisted of aqueous Sitagliptin/peptide solutions with the following ratios of 1:0, 1:426, 1:852, 1:1704 and 0:1 on a molar basis. Similarly, binary mixtures of peptides consisting of Ile-Pro-Ile-Gln-Tyr and another peptide (Trp-Lys, Trp-Pro, Trp-Arg or Trp-Leu) in the ratios of 1:0, 1:1, 2:1, 1:2 and 0:1 on a molar basis, were studied. The dose response for DPP-IV inhibition (n=3) was determined with each of the previous mixtures diluted in HPLC water at 7 different concentrations (Fig. 1 A & B).

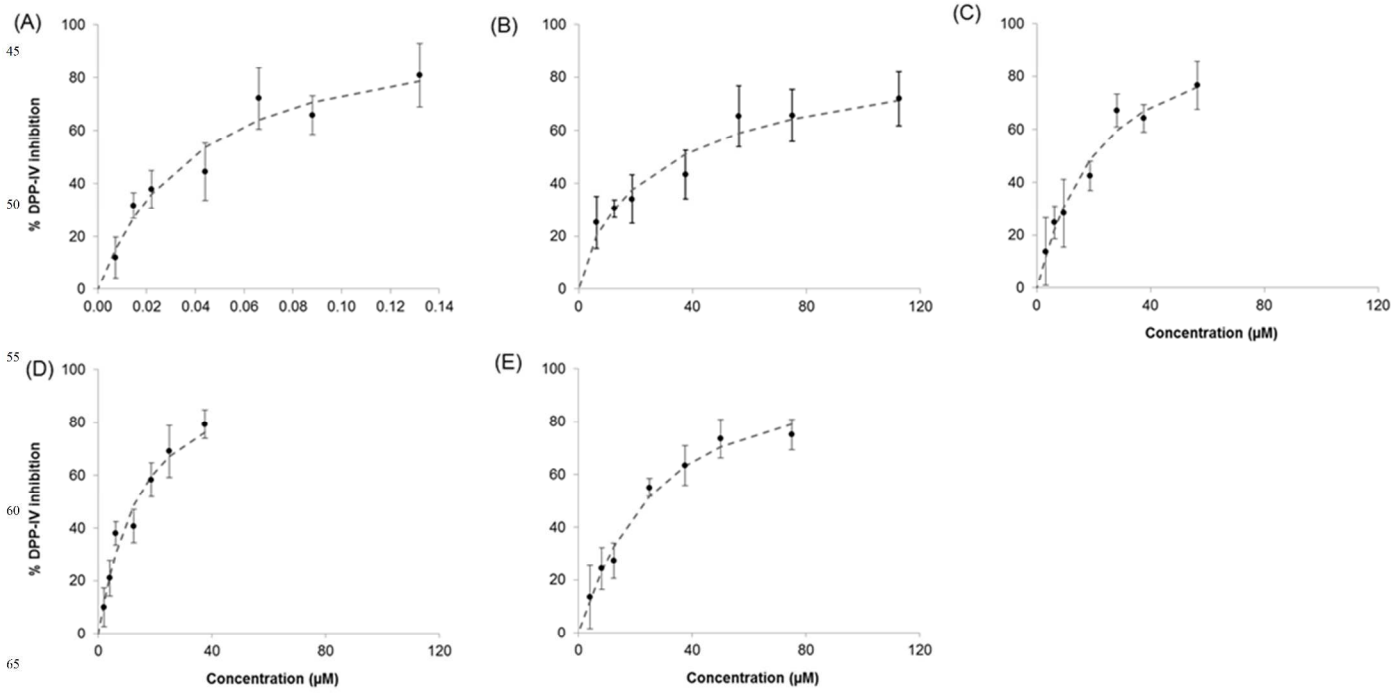

Fig 2: Dose response curves obtained for the dipeptidyl peptidase IV (DPP-IV) inhibitory effect of (A) Sitagliptin, (B) Trp-Lys and (C), (D) and (E) Sitagliptin: Trp-Lys (1:852, 1:426 and 1:1704 on a molar basis) mixtures, respectively. The individual points are the mean DPP-IV inhibition ± SD determined in triplicate (n=3).

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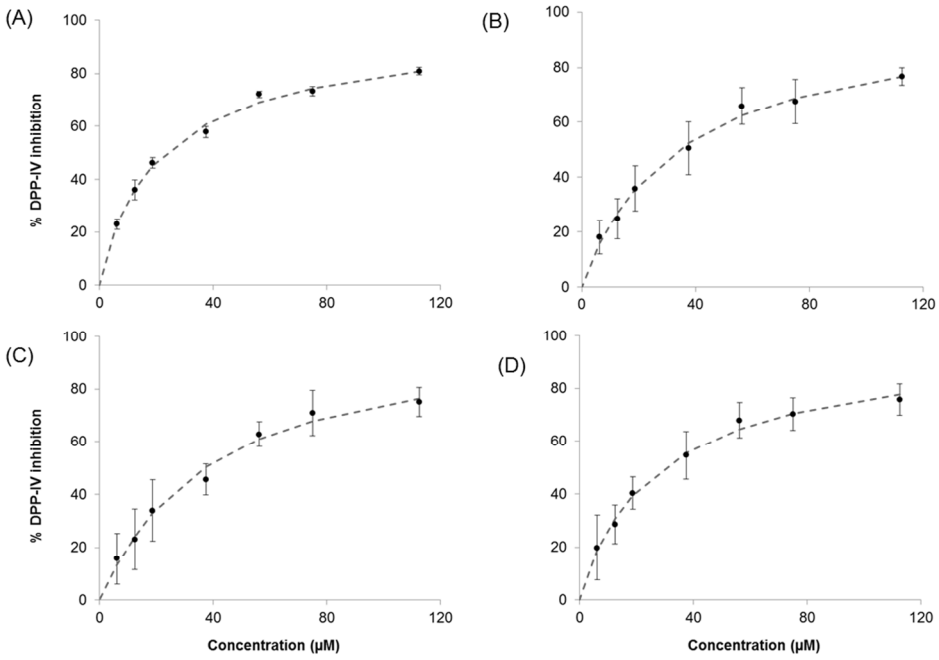

Fig. 3: Dose response curves obtained for the dipeptidyl peptidase IV (DPP-IV) inhibitory effect of (A) Ile-Pro-Ile-Gln-Tyr, (B), (C) and (D) binary Ile-Pro-Ile-Gln-Tyr:Trp-Lys (2:1, 1:1 and 1:2 on a molar basis) mixtures, respectively. The individual points are the mean DPP-IV inhibition ± SD determined in triplicate (n=3).

2.4. DPP-IV inhibition assay

The DPP-IV inhibition assay was carried out essentially as described by Nongonierma & FitzGerald [9]. Briefly, the Sitagliptin:peptide or binary peptide mixtures (25 µL) were pipetted onto a 96 well microplate (Sarstedt, Dublin, Ireland) 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}). The microplate was incubated at 37°C for 60 min in a microplate reader (Biotek Synergy HT, Winoosky, VT, USA) and absorbance of the released pNA was monitored at 405 nm. Each sample was analysed in triplicate (n=3). The half maximum inhibitory concentration (IC_{50}) for DPP-IV was determined by plotting the percentage inhibition as a function of the concentration of test compounds.

2.5. Determination of the isobole diagram at 50 % DPP-IV inhibition

The isobole diagrams for 50 % DPP-IV inhibition were plotted for the different Sitagliptin/peptide or binary peptide mixtures. Each isobole showed the IC_{50} value for the inhibitors on the x and y axes. The line between the two IC_{50} values corresponds to the line of additivity (Fig. 1C). The concentration addition (CA) effect is described by the following equation [12]:

$$CA = \frac{d_1}{IC_{50,1}} + \frac{d_2}{IC_{50,2}}$$

Where d_1 and d_2 are the concentrations of inhibitors 1 and 2, respectively, in a mixture yielding 50 % DPP-IV inhibition; $IC_{50,1}$ and $IC_{50,2}$ are the half maximum inhibitory concentrations of inhibitors 1 and 2, respectively.

The mixture of inhibitors 1 and 2 can have an additive (CA=1), synergistic (CA < 1) or antagonistic effect (CA > 1) on DPP-IV inhibition (Fig. 1C). The theoretical total additivity concentration (Zt) of the mixture was determined as described elsewhere [17] using an in-house Matlab program. Zt corresponds to the theoritical concentration of the mixture which should yield 50 % DPP-IV inhibition if the two inhibitors have an additive effect. Zt was calculated as follows:

$$Zt = \frac{IC_{50,1}}{p_1 + \frac{IC_{50,1}}{IC_{50,2}} \times p_2}$$

Where p_1 and p_2 are the proportions of inhibitors 1 and 2,

respectively; $IC_{50,1}$ and $IC_{50,2}$ are the half maximum inhibitory concentrations of inhibitors 1 and 2, respectively.

2.6. Statistical analysis

Means comparison was carried out with a one way ANOVA followed by a Student Newman-Keuls test using SPSS (version 22, SPSS Inc., Chicago, IL, USA) at a significance level $P < 0.05$. For each mixture, Zt was compared to the apparent IC_{50} value using a Student test ($P < 0.05$) as described elsewhere [12].

3. Results

3.1. Occurrence of the DPP-IV inhibitory peptides in 72 dietary food proteins

The five DPP-IV inhibitory peptides studied were found within 50% of the dietary proteins considered (supplementary Table S1). The *in silico* digestion of the dietary proteins predicted that 4 out of the 5 peptides may be released from 14 of the dietary proteins studied. It is interesting to note that 86% of these proteins are plant-derived. Although Trp-Pro was present within 16 of the proteins studied, it was not predicted to be released by gastrointestinal enzymes (Table 1). The outcome of the *in silico* analysis suggested that 4 of the target peptides may be released during the digestion of foods. Therefore, they may play a role in DPP-IV inhibition following oral ingestion.

3.2. Dose-response relationship for the Sitagliptin/peptide and the binary peptide mixtures

The five DPP-IV inhibitory peptides studied were selected based on differences in their mode of inhibition and the fact that they were relatively potent food protein-derived DPP-IV inhibitors (IC_{50} value < 60 μM) [8,18]. The IC_{50} values obtained during this study were of the same order as previously described [8,18] (Supplementary Table S2). Mixtures of Sitagliptin/peptides and binary peptides were evaluated for their ability to inhibit DPP-IV as outlined in section 2.4. The dose-response curves obtained for the Sitagliptin/Trp-Lys mixtures are illustrated on Fig. 2 and that for the binary peptide mixtures Ile-Pro-Ile-Gln-Tyr/Trp-Lys are shown on Fig. 3. A dose response relationship was seen with Sitagliptin and Ile-Pro-Ile-Gln-Tyr alone, and with all Sitagliptin/peptide and binary peptide mixtures (Fig. 2, 3 and data not shown).

Table 2: Theoretical additivity concentration (Zt) and apparent half maximum inhibitory concentration (IC_{50}) for the binary peptide and Sitagliptin/peptide mixtures. Values are mean ± confidence interval (P= 0.05) of triplicate determinations (n=3).

			Peptide				
			Ile-Pro-Ile-Gln-Tyr	**Trp-Lys**	**Trp-Pro**	**Trp-Arg**	**Trp-Leu**
Sitagliptin:peptide ratio (on a molar basis)[†]	1:1704	Zt (μM)	17.3 ± 0.7	22.5 ± 3.3	22.6 ± 1.3	22.6 ± 2.0	30.4 ± 2.0
		IC_{50} (μM)	18.9 ± 2.3[ns]	23.5 ± 1.5[ns]	22.6 ± 1.7[ns]	20.5 ± 2.6[ns]	28.3 ± 2.8[ns]
	1:852	Zt (μM)	13.8 ± 0.7	17.1 ± 2.7	16.9 ± 1.1	16.9 ± 1.2	21.12 ± 1.9
		IC_{50} (μM)	12.9 ± 0.6[*]	19.6 ± 2.4[ns]	18.4 ± 1.0[*]	16.2 ± 2.0[ns]	19.1 ± 1.3[ns]
	1:426	Zt (μM)	9.9 ± 0.8	11.5 ± 2.0	11.4 ± 1.0	11.4 ± 1.0	13.2 ± 1.5
		IC_{50} (μM)	8.8 ± 0.5[*]	13.3 ± 1.1[ns]	10.1 ± 1.3[ns]	11.3 ± 1.8[ns]	11.7 ± 1.0[ns]
Ile-Pro-Ile-Gln-Tyr:peptide ratio (on a molar basis)[†]	1:2	Zt (μM)	na	28.8 ± 2.5	29.5 ± 2.1	29.5 ± 2.5	37.9 ± 2.0
		IC_{50} (μM)	na	33.1 ± 5.4[ns]	27.2 ± 2.5[ns]	26.4 ± 4.0[ns]	45.2 ± 6.3[*]
	1:1	Zt (μM)	na	27.1 ± 2.0	27.6 ± 0.4	27.6 ± 1.8	32.8 ± 1.7
		IC_{50} (μM)	na	36.9 ± 6.3[*]	31.2 ± 3.6[ns]	32.2 ± 7.0[ns]	36.9 ± 5.3[ns]
	2:1	Zt (μM)	na	25.6 ± 1.7	26.1 ± 1.5	26.0 ± 1.3	28.9 ± 1.6
		IC_{50} (μM)	na	31.2 ± 1.1[*]	30.2 ± 6.2[ns]	29.0 ± 7.1[ns]	30.6 ± 2.0[ns]

[†]Values represent the mean of triplicate determination (n=3) of the theoretical additivity concentration (Zt) ± confidence interval (P=0.05) and the apparent half maximum inhibitory concentration (IC_{50}) ± confidence interval (P=0.05) for different Ile-Pro-Ile-Gln-Tyr:peptide (1:1, 1:2 and 2:1) and Sitagliptin:peptide (1:852, 1:426 and 1:1704) mixtures.

[ns]: the apparent IC_{50} value of the mixture is not significantly different from Zt ($P > 0.05$)

*: the apparent IC_{50} value of the mixture is significantly different from Zt ($P < 0.05$)

na: not applicable

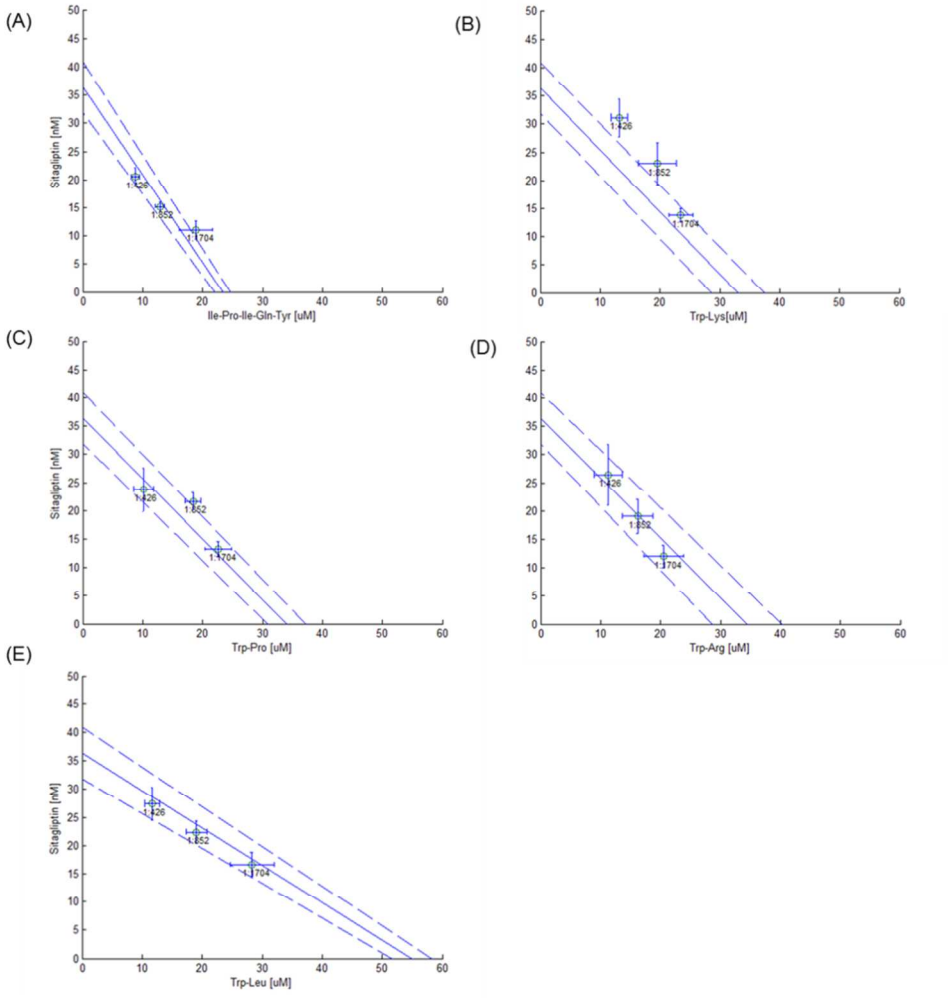

Fig 4: Isobole diagram obtained at 50 % dipeptidyl peptidase IV (DPP-IV) inhibition (IC_{50}) for different Sitagliptin:peptide (1:0, 0:1, 1:852, 1:426 and 1:1704 on a molar basis) mixtures. Each point represents the IC_{50} ± confidence interval (*P=0.05*). The peptides tested are (A) Ile-Pro-Ile-Gln-Tyr, (B) Trp-Lys, (C) Trp-Pro, (D) Trp-Arg and (E) Trp-Leu.

3.3. Sitagliptin-peptide and peptide-peptide interactions

The 50 % isobole diagram shows the IC_{50} value for Sitagliptin or Ile-Pro-Ile-Gln-Tyr on the y axis and that of the peptide on the x axis (Fig. 4 & 5). In a few instances, the apparent IC_{50} value for the mixture was close to the line of additivity for Sitagliptin:Ile-Pro-Ile-Gln-Tyr and Sitagliptin:Trp-Pro (1:426 and 1:852), Sitagliptin:Trp-Arg and Sitagliptin:Trp-Leu (1:426, 1:852 and 1:1704), Ile-Pro-Ile-Gln-Tyr:Trp-Arg and Ile-Pro-Ile-Gln-Tyr:Trp-Pro (1:2 and 2:1) and Ile-Pro-Ile-Gln-Tyr:Trp-Leu (2:1). For the other mixtures, the values were either in the area of the isobole corresponding to an antagonistic effect or in the area corresponding to a synergistic effect.

Most Zt values were not significantly different ($P > 0.05$) from the apparent IC_{50} value (Table 2), suggesting an additive effect of the mixture on DPP-IV inhibition. However, three Sitagliptin/peptide mixtures (Sitagliptin:Ile-Pro-Ile-Gln-Tyr (1:426 and 1:852) and Sitagliptin:Trp-Pro (1:852)) had apparent IC_{50} values which were significantly lower ($P < 0.05$) from that of Zt (12.9 vs. 13.8, 8.8 vs. 9.9 and 18.4 vs. 16.9 µM, respectively), indicating a synergistic effect for the Sitagliptin:Ile-Pro-Ile-Gln-Tyr mixtures and an antagonistic effect for the Sitagliptin:Trp-Pro mixture on DPP-IV inhibition. Similarly, three binary peptide mixtures (Ile-Pro-Ile-Gln-Tyr:Trp-Lys (1:1 and 2:1) and Ile-Pro-Ile-Gln-Tyr:Trp-Leu (1:2)) had apparent IC_{50} values significantly higher than that of Zt (36.9 vs. 27.3; 31.2 vs. 25.8 and 45.2 vs. 37.8 µM, respectively), also suggesting an antagonistic effect of the binary peptide mixture on DPP-IV inhibition.

4. Discussion

Confirmatory studies conducted with synthetic peptides, following mass spectrometric identification frequently show that several peptide sequences identified within active fractions of food protein hydrolysates display DPP-IV inhibitory properties [5,6,10,19]. This indicates that the overall DPP-IV inhibitory effect seen in food protein hydrolysates originates from a mixture of peptides rather than a single peptide. The isobole methodology has been mainly utilised to study interactive effects between

drugs, fertilisers, pesticides and phytochemicals [13] with a limited number of examples applied to antimicrobial peptide mixtures [20,21]. An additive effect of Sitagliptin (when studied at one level) and peptide mixtures on DPP-IV inhibitory properties has previously been shown [11]. However, to our knowledge, study of the effect of drug-peptide and binary peptide mixtures on DPP-IV inhibition has not previously been described using an isobolographic approach.

The synthetic substrate, Gy-Pro-pNA, used herein for the DPP-IV inhibitory assay has a different N-terminal amino acid sequence than that of the incretins (His-Ala for GPL-1 and Tyr-Ala for GIP). However, in the case of the synthetic substrate and the incretins, the presence of a Pro or Ala at position P1 is consistent with the sequence of DPP-IV preferred substrates [22,23]. Therefore, the results described herein may be extrapolated to a physiological situation where food protein-derived peptides may inhibit DPP-IV, preventing incretin degradation.

Most Sitagliptin/peptide and binary peptide mixtures showed an additive effect (Table 2 and Fig. 4 & 5). However, the Sitagliptin:Trp-Pro (1:852) mixture showed an antagonistic effect on DPP-IV inhibition. The extent of apparent IC_{50} increase compared to Zt was 9 % for the Sitagliptin:Trp-Pro (1:852) mixture. In the case of the Sitagliptin:Ile-Pro-Ile-Gln-Tyr (1:426 and 1:852) mixtures, a synergistic effect was seen with a reduction of the IC_{50} value compared to Zt of 7 and 11 %, respectively. Although the peptides studied have different modes of inhibition (competitive, non-competitive, true or substrate-type inhibitor), there did not seem to be a clear trend showing specific types of interactions in the mixtures in one instance or the other. However, it is interesting to note that, the synergistic effect was seen with a mixture of competitive DPP-IV inhibitors (Sitagliptin and Ile-Pro-Ile-Gln-Tyr). While most antagonistic effects involved a non-competitive DPP-IV inhibitor (Trp-Lys and Trp-Pro). In addition, it was not clear why the antagonistic effect was only seen for certain ratios of the DPP-IV inhibitors studied (Table 2). A number of *in silico* approaches have suggested that non-competitive DPP-IV inhibitors may interact at a secondary binding site located in the neighbourhood of the active site [24,25]. Binding of non-competitive inhibitors to a secondary binding site may in some instance restrict access to the active site for competitive DPP-IV inhibitors.

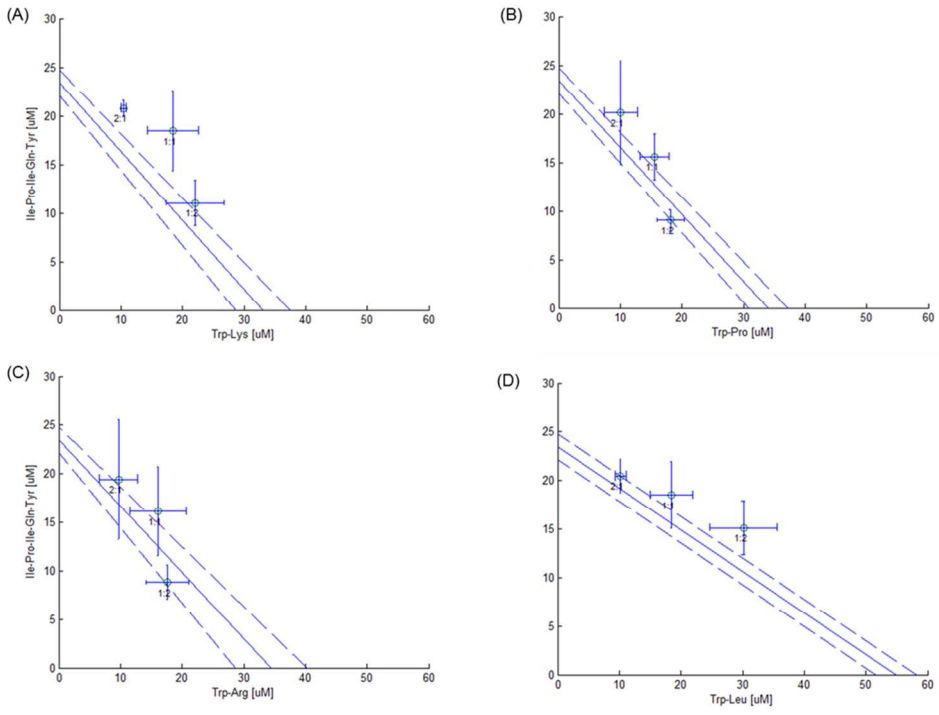

Fig 5: Isobole diagram obtained at 50 % dipeptidyl peptidase IV (DPP-IV) inhibition (IC_{50}) for binary Ile-Pro-Ile-Gln-Tyr:peptide (1:0, 0:1, 1:1, 1:2 and 2:1) mixtures. Each point represents the IC_{50} ± confidence interval (*P=0.05*). The peptides tested were (A) Trp-Lys, (B) Trp-Pro, (C) Trp-Arg and (D) Trp-Leu.

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Ile-Pro-Ile-Gln-Tyr behaves like a substrate type DPP-IV inhibitor [8]. This may explain the overall increase of DPP-IV inhibition seen in the Sitagliptin:Ile-Pro-Ile-Gln-Tyr (1:852 and 2:426) mixtures. Trp-Lys is an hydrophilic and positively charged peptide, while Ile-Pro-Ile-Gln-Tyr (pI 5.5) is negatively charged at the assay pH (8.0). It may be possible that some electrostatic interactions between Trp-Lys and Ile-Pro-Ile-Gln-Tyr may have reduced the amount of inhibitors available for DPP-IV inhibition. Surprisingly, no antagonistic effect was seen with Trp-Arg, which has very similar characteristic to Trp-Lys. An antagonistic effect was also seen in the Ile-Pro-Ile-Gln-Tyr:Trp-Leu (1:2) mixture. Both peptides are competitive DPP-IV inhibitors and compete for binding at the same site on DPP-IV. This may explain why an antagonistic effect was seen when Trp-Leu was present at the highest concentration.

The antagonistic activity of peptide mixtures on DPP-IV inhibition could result in the activity of specific peptides being "masked" by the presence of other peptides. This may have consequences in particular in bioassay driven fractionation approaches where specific fractions may be disregarded even though they contain relatively potent DPP-IV inhibitory peptides. Similar results have been described where the immunomodulatory properties of an hydrolysate was less than that of its associated isoelectric focusing fractions when tested at the same concentration [26]. This was explained by the fact that some peptides may interact through physicochemical interactions [27], making them unavailable as bioactive components.

A well-known example of a food drug interaction is the combination of grape fruit juice and drugs. Furanocoumarin from grapefruit juice has been shown to inhibit the drug metabolising enzyme, cytochrome P450 (CYP) 34A [28]. In terms of antidiabetic activity, small animal studies have demonstrated that the ingestion of Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu (β-casein f70-77, DPP-IV IC_{50} = 160 μM) or a tryptic β-lactoglobulin hydrolysate containing Val-Ala-Gly-Thr-Trp-Tyr (β-lg f15-20, DPP-IV IC_{50} = 174 μM) could lower plasma glucose following an oral glucose tolerance test [5,6]. Recently, it was shown that a porcine skin gelatin hydrolysate could inhibit plasma DPP-IV in rats as well as reducing serum glucose in the post prandial phase [29]. However, little or no data appears to exist on the effect of foods on the pharmacokinetics of Sitagliptin *in vivo* following food intake [30]. There is therefore a need to evaluate the peptide sequences studied herein in humans to assess their *in vivo* biological activity. The interactions reported with the Sitaglitpin/peptide mixtures suggest that it may be possible to lower drug intake level when combined with food protein-derived DPP-IV inhibitory peptides. This may help to reduce the possible side-effects associated with drug intake [31].

Conclusion

A systematic approach has been utilised to study the effect of Sitagliptin/peptide and binary peptide mixtures on DPP-IV inhibition using an isobole methodology. It was shown in most cases that there was an additive effect of the mixtures on overall DPP-IV inhibition. However, in some instances antagonistic or synergistic effects were observed. Since the ability of food protein-derived peptides to inhibit DPP-IV has been demonstrated *in vitro*, the interactive effects described herein may therefore be relevant to the post-prandial regulation of serum glucose and to the pharmacokinetics of antidiabetic drugs. In addition, the isobolographic approach used herein may aid in the formulation of foods with a desired DPP-IV inhibitory profile which in turn may complement the effects of T2D preventative and therapeutic agents. *In vivo* studies are required to test these hypotheses.

Acknowledgements

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Notes and references

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Supplementary data

Table S1: *In silico* analysis showing the occurrence of Trp-Lys, Trp-Pro, Trp-Arg, Trp-Leu and Ile-Pro-Ile-Gln-Tyr in food proteins.

			Peptide occurrence in proteins‡				
Origin	**Protein***	**Accession number†**	**Ile-Pro-Ile-Gln-Tyr**	**Trp-Arg**	**Trp-Lys**	**Trp-Leu**	**Trp-Pro**
Wheat (*Triticum aestivum*)	α/ β-gliadin	P02863	0	0	0	0	0
	Glutenin, high molecular weight subunit 12	P08488	0	0	0	0	0
	Glutenin, low molecular weight subunit 1D1	P10386	0	0	0	0	0
	Large subunit RuBisCO	P11383	0	1	1	0	0
	Small subunit RuBisCO	P26667	0	0	1	0	1
Barley (*Hordeum vulgare*)	B hordein	Q40026	0	0	0	0	1
	D hordein	Q40054	0	0	0	0	0
	γ-hordein-3	P80198	0	0	0	0	0
	Large subunit RuBisCO	P05698	0	1	1	0	0
	Small subunit RuBisCO	Q40004	0	0	1	0	1
Oat (*Avena sativa*)	Avenin	P27919	0	1	2	0	1
	11S globulin	Q38779	0	0	0	0	0
	12S globulin	Q49257	0	0	0	0	1
	Large subunit RuBisCO	P48684	0	1	1	0	0
	Small subunit RuBisCO	Q9ZWG4	0	0	1	0	1
Corn (*Zea mays*)	Large subunit RuBisCO	P00874	0	1	1	0	0
	Small subunit RuBisCO	P05348	0	0	1	0	1
Rice (*Oryza sativa* subsp. Japonica)	Large subunit RuBisCO	P0C512	0	1	1	0	0
	Small subunit RuBisCO	P18566	0	0	1	0	1
Sorghum (*Sorghum vulgare*)	Large subunit RuBisCO	A1E9T2	0	1	1	0	0
	Small subunit RuBisCO	C5Y519	0	0	1	0	1
Soybean (*Glycine hispida*)	Basic 7S	P13917	0	1	0	0	0
	Glycinin	P04347	0	0	0	0	0
	β-conglycinin, β-chain	P25974	0	0	0	0	0
	β-conglycinin, α-chain	P13916	0	0	0	0	1
	β-conglycinin, α'-chain	P11827	0	0	0	0	0
Quinoa (*Chenopodium quinoa*)	RuBisCO large chain	K4P448	0	1	0	0	0
Canola (*Brassica napus*)	Cruciferin	P11090	0	0	0	0	0
	Napin small chain	P17333	0	0	0	1	0
	Napin large chain	P17333	0	0	0	0	0
Amaranth (*Amaranthus hypochondriacus*)	Large subunit RuBisCO	P16306	0	1	1	0	0
Palamaria palmata (*Rhodymenia palmata*)	50S ribosomal protein L31, chloroplastic	Q5MIM5	0	0	0	0	0
	PALPL Ribosomal protein 12	Q5MIL8	0	0	0	0	0
	Allophycocyanin α chain	M1UZ22	0	1	1	0	0
	Allophycocyanin β chain	M1VJV1	0	1	0	0	0
	Phycocyanin α	I2FJU0	0	1	1	0	0
	Phycocyanin β	I2FJT9	0	0	1	0	1
	Phycoerythrin α subunit	F2ZAL8	0	0	0	0	0
	Phycoerythrin β subunit	F2ZAL7	0	0	0	0	0
	RuBisCO large chain	Q9THF8	0	0	0	0	0
	RuBisCO small chain	O98734	0	0	0	0	0
	Small subunit RuBisCO	Q9XGX5	0	0	1	0	1
Chicken egg (*Gallus gallus*)	Ovalbumin	P01012	0	0	0	0	0
	Ovotransferrin	P02789	0	0	0	0	0
	Ovomucoid	P01005	0	0	0	0	0
Bovine milk (*Bos taurus*)	α_{s1}-casein	P02662	0	0	0	0	0
	α_{s2}-casein	P02663	0	0	0	0	0
	β-casein	P02666	0	0	0	0	0

	κ-casein	P02668	1	0	0	0	0
	β-lactoglobulin	P02754	0	0	0	0	0
	α-lactalbumin	P00711	0	0	0	2	0
	Lactoferrin	P24627	0	1	1	0	0
	BSA	P02769	0	0	0	0	0
Bovine meat (*Bos taurus*)	Myosin-1	Q9BE40	0	1	1	2	1
	Myosin regulatory light chain 12B	A4IF97	0	0	0	0	0
	Actin, cytoplasmin 1	P60712	0	0	0	0	0
	Collagen α-1 (III) chain	P02459	0	0	1	0	0
Pig (*Sus scrofa*)	β-actin	Q8SPK6	0	0	0	0	0
	Actin, α skeletal muscle	P68137	0	0	0	0	0
	Myosin light chain	Q29069	0	0	0	0	0
	Myosin heavy chain	Q29623	0	0	0	1	0
Atlantic salmon (*Salmo salar*)	Actin, cytoplasmic 1	O42161	0	0	0	0	0
	Myosin regulatory light chain 2	Q7ZZN0	0	0	0	0	0
	Slow myosin heavy chain	Q2HXU3	0	0	0	1	0
	Collagen Type XI α2	A7KE05	0	1	0	0	0
Chum salmon (*Oncorhynchus keta*)	Type 1 collagen α2 chain	Q8UUJ4	0	0	0	0	0
	Actin	Q9PVN1	0	0	0	0	0
	β-actin	J7ID80	0	0	0	0	0
	Myosin heavy chain	Q8JIP5	0	2	1	2	1
Tuna (*Thunnus orientalis*)	β-actin	A9CM08	0	0	0	0	0
	Myosin heavy chain-1	G9M5T1	0	1	1	2	1
	Myosin heavy chain-2	G9M5T2	0	1	1	2	1

*RuBisCO: Ribulose bisphosphate carboxylase; BSA bovine serum albumin

†Accession number from UniProt database, data presented within this table is relative to the mature protein sequence

‡0: peptide not found within the protein sequence; 1 and 2: peptide found once or twice, respectively, within the protein sequence

Table S2: Inhibitory concentration inducing 50 % inhibition (IC_{50}) for dipeptidyl peptidase IV (DPP-IV) and type of inhibition as determined by Lineweaver and Burk analysis.

Compound	IC_{50} (μM)*	Type of inhibition‡
Sitagliptin	0.037 ± 0.009^{a}	competitive
Ile-Pro-Ile	3.6 ± 0.6^{b}	substrate-type, competitive
Ile-Pro-Ile-Gln-Tyr	23.3 ± 1.4^{c}	substrate-type, competitive
Trp-Lys	33.1 ± 4.0^{d}	non-competitive
Trp-Pro	33.3 ± 2.8^{d}	non-competitive
Trp-Arg	34.9 ± 6.0^{d}	non-competitive
Trp-Leu	53.9 ± 2.4^{e}	true, competitive

*Values represent the mean half maximum inhibitory concentration (IC_{50}) ± confidence interval (P=0.05). Values with different superscript letters are significantly different ($P < 0.05$)

‡Type of DPP-IV inhibition as reported elsewhere [18,32]

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Utilisation of the isobole methodology to study dietary peptide-drug and peptide-peptide interactive effects on dipeptidyl peptidase IV (DPP-IV) inhibition

Alice B. Nongonierma[a] & Richard J. FitzGerald*[a]

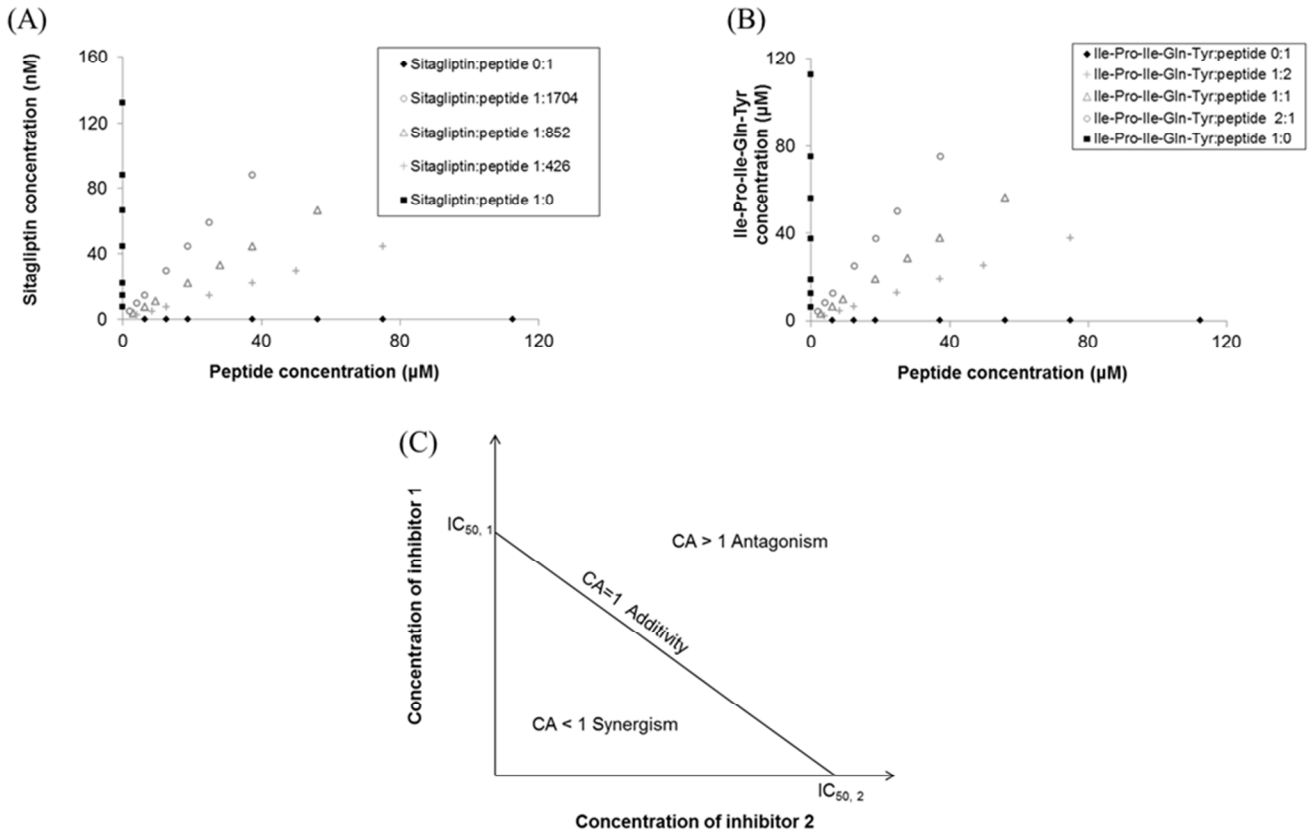

Fig 1: Experimental design used to study the dose response effect of (A) different Sitagliptin/peptide (Ile-Pro-Ile-Gln-Tyr, Trp-Lys, Trp-Pro, Trp-Arg and Trp-Leu) and (B) Ile-Pro-Ile-Gln-Tyr:peptide (Trp-Lys, Trp-Pro, Trp-Arg and Trp-Leu) mixtures on dipeptidyl peptidase IV (DPP-IV) inhibition. (C) Schematic representation of a 50% inhibition isobole diagram and interpretation of the type of interactions between two inhibitors based on the concentration addition (CA) value of the mixture. IC_{50}: half maximum inhibitory concentration.

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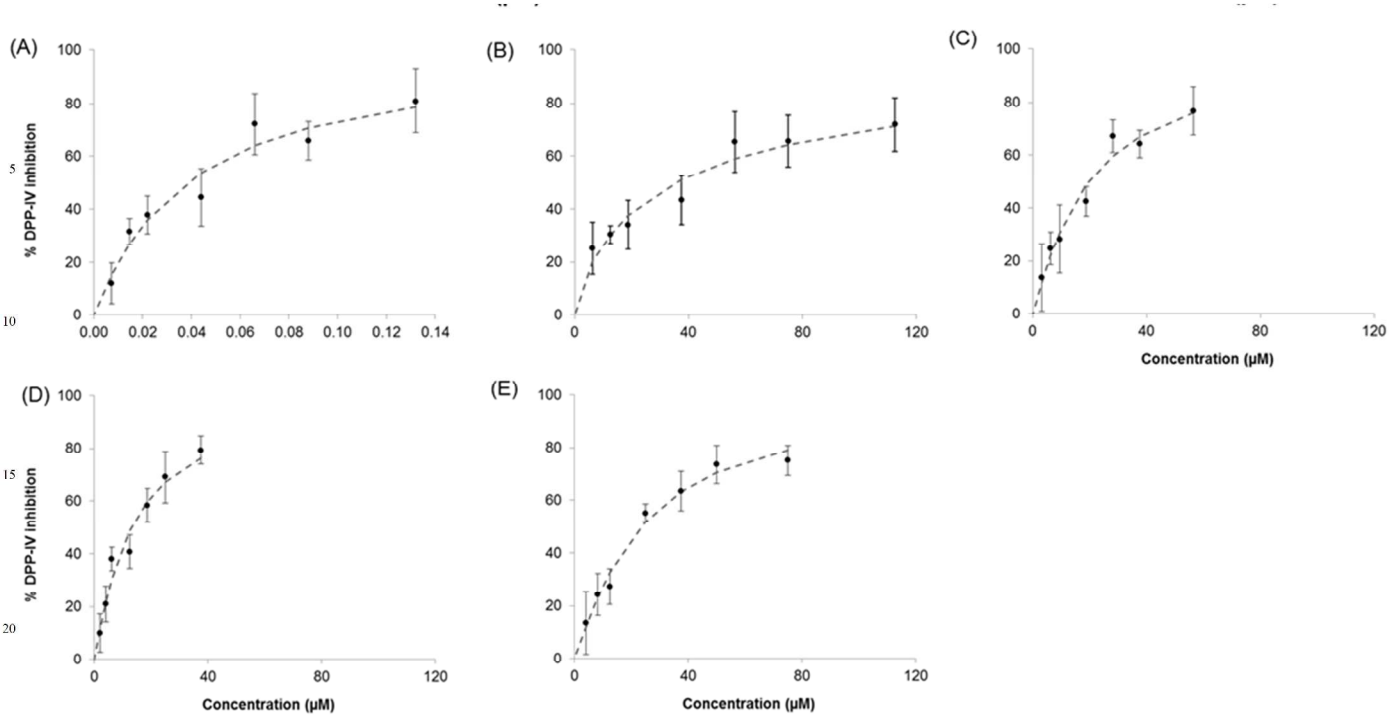

Fig 2: Dose response curves obtained for the dipeptidyl peptidase IV (DPP-IV) inhibitory effect of (A) Sitagliptin, (B) Trp-Lys and (C), (D) and (E) Sitagliptin: Trp-Lys (1:852, 1:426 and 1:1704 on a molar basis) mixtures, respectively. The individual points are the mean DPP-IV inhibition ± SD determined in triplicate (n=3).

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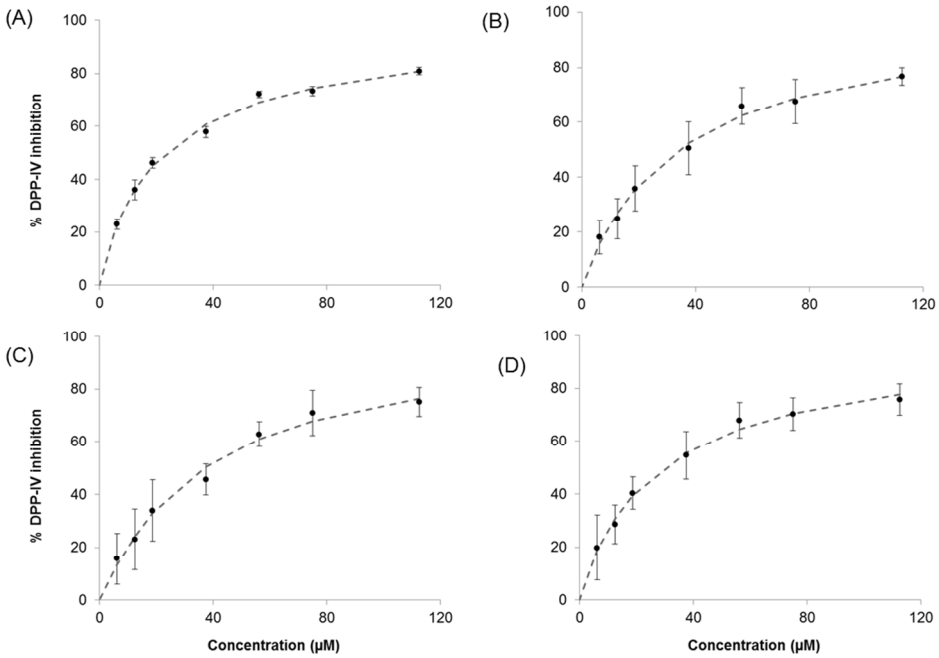

Fig. 3: Dose response curves obtained for the dipeptidyl peptidase IV (DPP-IV) inhibitory effect of (A) Ile-Pro-Ile-Gln-Tyr, (B), (C) and (D) binary Ile-Pro-Ile-Gln-Tyr:Trp-Lys (2:1, 1:1 and 1:2 on a molar basis) mixtures, respectively. The individual points are the mean DPP-IV inhibition ± SD determined in triplicate (n=3).

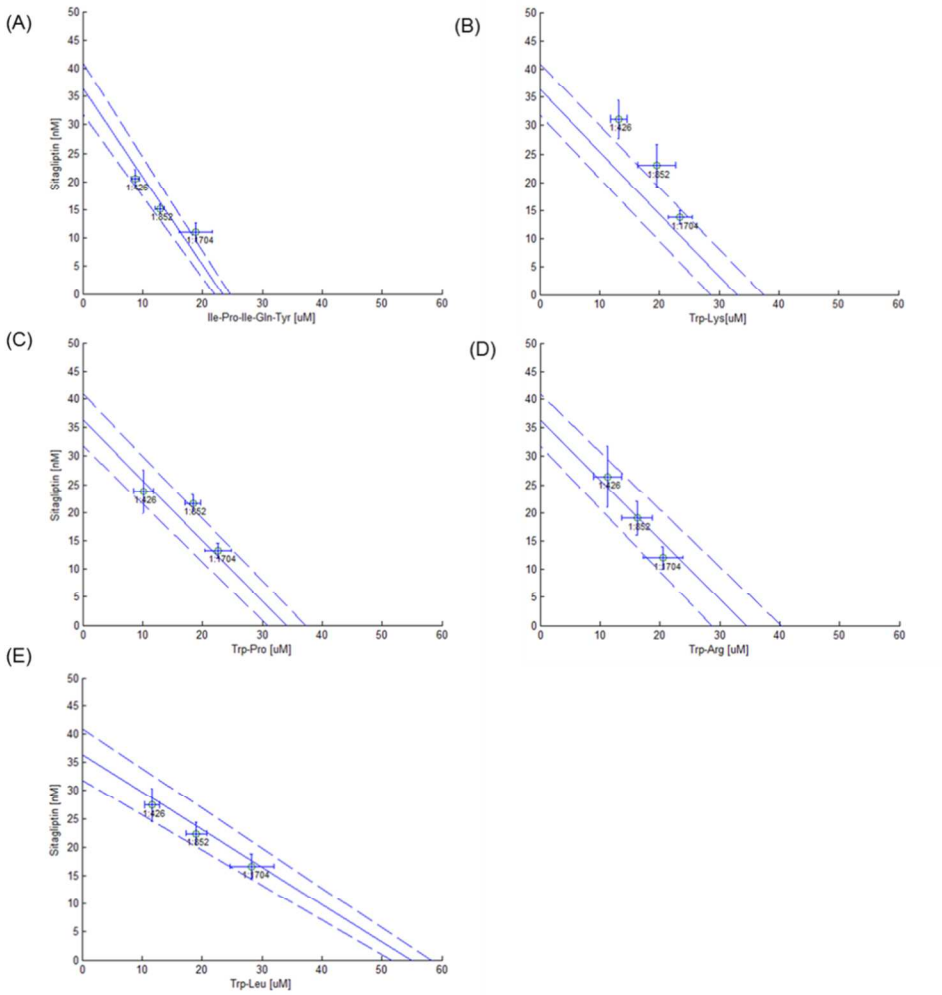

Fig 4: Isobole diagram obtained at 50 % dipeptidyl peptidase IV (DPP-IV) inhibition (IC_{50}) for different Sitagliptin:peptide (1:0, 0:1, 1:852, 1:426 and 1:1704 on a molar basis) mixtures. Each point represents the IC_{50} ± confidence interval (*P=0.05*). The peptides tested are (A) Ile-Pro-Ile-Gln-Tyr, (B) Trp-Lys, (C) Trp-Pro, (D) Trp-Arg and (E) Trp-Leu.

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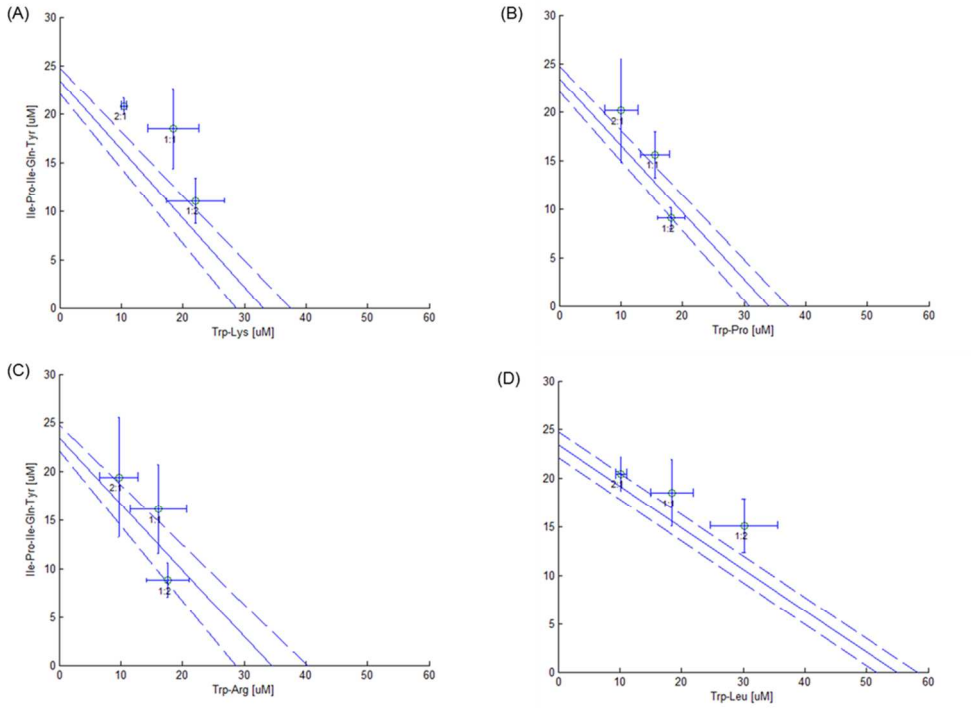


Fig 5: Isobole diagram obtained at 50 % dipeptidyl peptidase IV (DPP-IV) inhibition (IC_{50}) for binary Ile-Pro-Ile-Gln-Tyr:peptide (1:0, 0:1, 1:1, 1:2 and 2:1) mixtures. Each point represents the IC_{50} ± confidence interval (*P=0.05*). The peptides tested were (A) Trp-Lys, (B) Trp-Pro, (C) Trp-Arg and (D) Trp-Leu.

Food protein substrates

↓

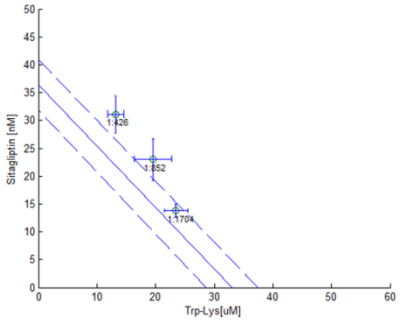
***In silico* gastrointestinal digestion** → **Ile-Pro-Ile-Gln-Tyr, Trp-Lys, Trp-Pro, Trp-Arg & Trp-Leu**

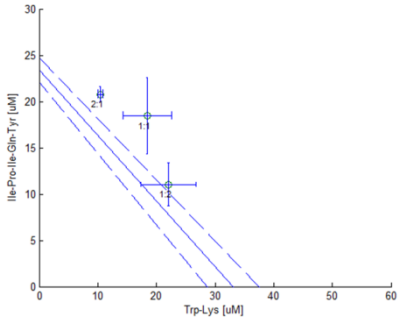
↓

- DPP-IV IC_{50} < 60 µM
- Different modes of inhibition

↓

Isobole methodology to study binary peptide and Sitagliptin/peptide mixtures





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Additive, antagonistic and synergistic effects seen with different binary peptide and Sitagliptin/peptide mixtures

Peptide and Sitagliptin®/peptide mixtures may enhance DPP-IV inhibition. Food protein-derived peptides may complement the action of antidiabetic drugs.