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Article

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A General Method for Making Peptide Therapeutics Resistant to Serine Protease Degradation; Application to Dipeptidyl Peptidase IV Substrates

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KEYWORDS: alpha-lytic protease (αLP), amino acid analogue, brain natriuretic peptide (BNP), chymotrypsin, diabetes, dipeptidyl peptidase 8 (DPP8), dipeptidyl peptidase IV (DPP IV), enterostatin (ENT), fibroblast activation protein alpha (FAPα), glucagon-like peptide-1 (7-36) amide (GLP-1), glucose-dependent insulinotropic peptide (GIP), half-life ($t_{½}$), neuropeptide Y (NPY), oxyntomodulin (OXM), peptide therapeutics, protease, serine protease, trypsin

Abstract

Bioactive peptides have evolved to optimally fulfill specific biological functions -- a fact which has long attracted attention for their use as therapeutic agents. While there have been some recent commercial successes fostered in part by advances in large-scale peptide synthesis, development of peptides as therapeutic agents has been significantly impeded by their inherent susceptibility to protease degradation in the bloodstream. Here we report that incorporation of specially designed amino acid analogues at the P1' position, directly C-terminal of the enzyme cleavage site, renders peptides, including glucagon-like peptide-1 (7-36) amide (GLP-1) and six other examples, highly resistant to serine protease degradation without significant alteration of their biological activity. We demonstrate the applicability of the method to a variety of proteases, including dipeptidyl peptidase IV (DPP IV), dipeptidyl peptidase 8 (DPP8), fibroblast activation protein alpha (FAPα), alpha-lytic protease (αLP), trypsin, and chymotrypsin. In summary, the "P1' modification" represents a simple, general, and highly adaptable method of generating enzymatically stable peptide-based therapeutics.

INTRODUCTION

Naturally occurring bioactive peptides offer a number of advantages as therapeutic agents over synthetic small molecules or antibodies.[1] Honed over eons by evolution to have a particular function, usually through specific interaction with one or more targets, peptides are relatively free of adverse events caused by cross-reactivity with unintended targets. Owing to their smaller size, peptides also provide superior tissue penetration than antibodies.[1] Advances in peptide chemistry and manufacturing have fostered the clinical development and application of therapeutic peptides. In fact, the number of new peptides entering clinical testing has nearly doubled in the past decade.[2] The emergence of peptides as mainstream therapeutic agents has been hindered, however, by their susceptibility to proteolytic degradation and inactivation in circulation.

The serine protease dipeptidyl peptidase IV (DPP IV) preferentially cleaves peptide bonds after Pro or Ala residues penultimate to the N-terminus[3-6] and functions as the principal determinant of the circulating half-life for many bioactive peptides. Several endogenous DPP IV substrates, including glucagon-like peptide-1 (7-36) amide (GLP-1) ($t_{½}$ < 2 min)[3], glucose-dependent insulinotropic factor (GIP) ($t_{½}$= 5-7 min)[4], oxyntomodulin (OXM) ($t_{½}$ = 12 min)[7], neuropeptide Y (NPY) ($t_{½}$ = 12 min)[8], brain natriuretic peptide (BNP) ($t_{½}$ = 15-20 min)[9], and enterostatin (ENT) ($t_{½}$ = 5 min)[10], have biological properties that render them therapeutically useful. For example, the numerous glucose-lowering properties of the incretin hormone GLP-1 make it an ideal agent for the treatment of Type 2 diabetes mellitus (T2D).[11-13] Similar to GLP-1, the hormones GIP and OXM act as glucose-lowering agents and have been studied extensively as potential treatments for T2D.[11, 14-20] Furthermore, OXM and ENT suppress appetite and enhance energy expenditure and have therefore been implicated as potential treatments for obesity. [21-23]

NPY, which possesses potent anxiolytic and anti-depressant effects, has been proposed as a therapeutic agent for anxiety, depression, and post-traumatic stress disorder.[24-26] BNP, which plays an important role in the body's natural defense against hypertension and plasma volume expansion, is currently FDA-approved for the treatment of acutely decompensated congestive heart failure.[27, 28] With such short half-lives, however, therapeutic use of these peptides would require multiple injections per day or intravenous infusion. Such methods of administration are expensive, inconvenient, and invasive; rendering them impractical for the treatment of chronic diseases that require daily medication.

Here we report that substitution of the P1' residue, which lies directly C-terminal of the enzyme cleavage site, with an amino acid analogue containing a tertiary-substituted β-carbon atom renders these peptides resistant to proteolytic degradation by DPP IV without significantly altering their biological activity. Circular dichroism and receptor activation studies indicate that the modified peptide analogues retain the overall secondary structure and receptor agonist activity of their native counterparts. Furthermore, experiments in diabetic (*db*/*db*) mice revealed that treatment with a P1'-modified version of the naturally-occurring hormone GLP-1 elicited a more potent and prolonged glucose-lowering affect than treatment with the native peptide. The "P1' modification" appears to confer resistance to essentially all serine proteases, as evidenced by our studies with DPP IV, dipeptidyl peptidase 8 (DPP8), fibroblast activation protein alpha (FAPα), alpha-lytic protease (αLP), chymotrypsin, and trypsin. The modification also confers resistance to the metalloprotease aminopeptidase P 2 (APP2), suggesting that it may effectively block proteolysis by this class of enzyme as well. Kinetic studies suggest that the P1' modification prevents degradation by blocking the enzyme's catalytic machinery rather than by interfering with substrate recognition or binding. In summary, the P1' modification represents a

general and adaptable method of generating enzymatically stable peptides that retain the physiochemical properties and biological activity of their native counterparts.

RESULTS

Amino Acid Analogues. Three hexapeptides with the sequence APXSWS, where X was Leu, Ile, or *tert*-Leu (**1**), were designed to investigate the substrate specificity of DPP IV at the P1' position. The amino acid analogue **1** is an isomer of the amino acids Leu and Ile that contains a tertiary-substituted β-carbon; a stereochemical arrangement that is not present in any naturally occurring amino acid (Figure 1). As shown in Figure 2, the synthetic hexapeptides containing the natural amino acid residues Leu or Ile at the P1' positions were quickly hydrolyzed by DPP IV. At 30 min, less than 70% of AP(Leu)SWS and AP(Ile)SWS remained intact. In contrast, the hexapeptide with the amino acid analogue **1** at the P1' position was completely resistant to DPP IV-mediated cleavage over the 30 min incubation.

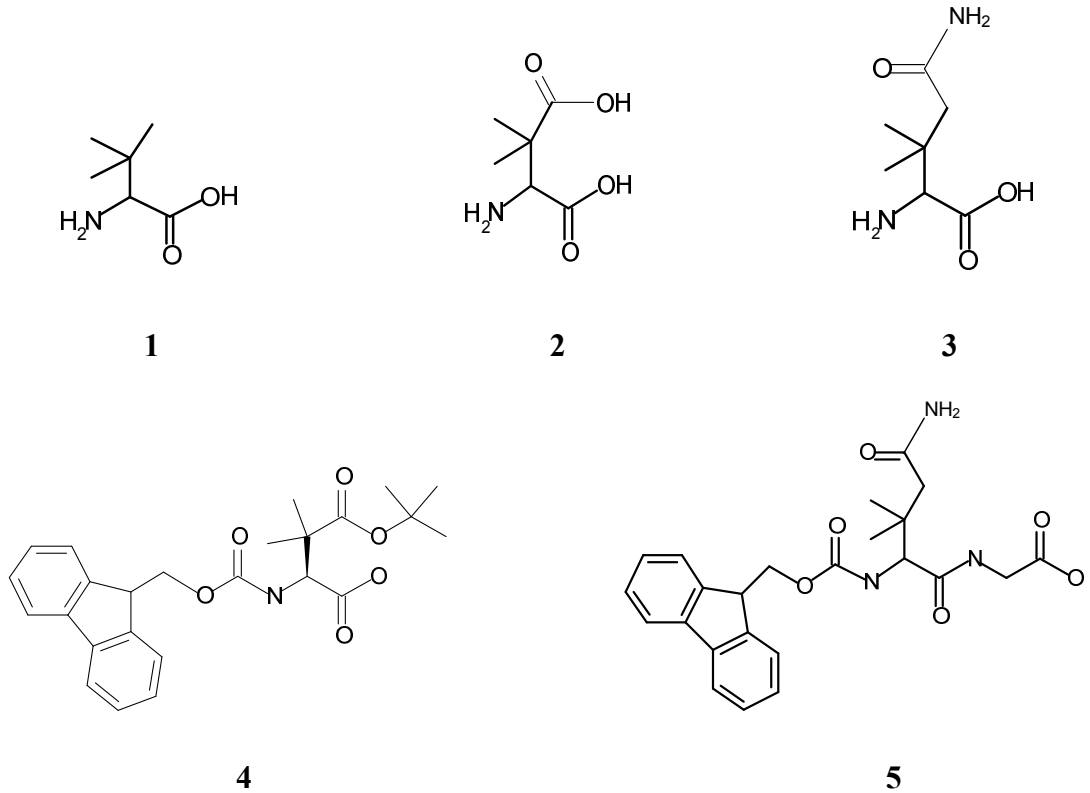

Figure 1. Structures of the amino acid analogues *tert*-Leu (**1**), β-dimethyl-Asp (**2**), β-dimethyl-Gln (**3**), N-Fmoc-L-β, β-dimethyl-Asp(*t*-butyl) (**4**), and N-Fmoc-L-β, β-dimethyl-Gln-Gly(*t*-butyl)-OH (**5**)

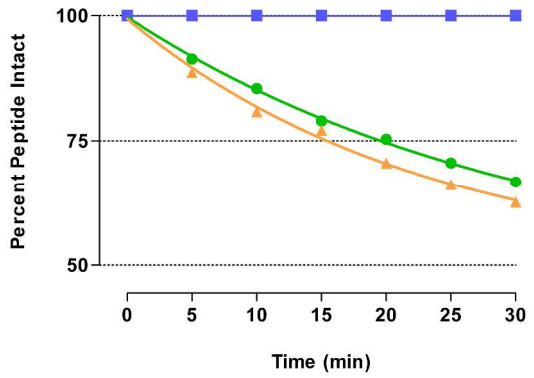

Figure 2. Degradation of synthetic substrates by DPP IV. AP(Leu)SWS (●), AP(Ile)SWS (▲), and AP(**1**)SWS (■) were incubated with purified porcine DPP IV (10 mU/mL) at 37 °C. The amount of intact peptide was quantified by HPLC-MS and expressed as a percentage of the corresponding value observed before exposure to enzyme digestion (time = 0).

Subsequent to this finding, the amino acid analogues β-dimethyl-Asp (**2**) and β-dimethyl-Gln (**3**) were specially designed in our laboratory to function as tertiary-substituted β-carbon atom containing analogues of the naturally occurring amino acid residues Asp and Gln, respectively (Figure 1). N-Fmoc-L-β, β-dimethyl-Asp(*t*-butyl) (**4**) and N-Fmoc-L-β, β-dimethyl-Gln-Gly(*t*-butyl)-OH (**5**) were synthesized as protected forms of **2** and **3**, respectively, for incorporation into protease substrate analogues (Figure 1 and Supporting Information).

Protease-resistant GLP-1 Analogues. *In Vitro Degradation*. The P1' Glu residue of the endogenous DPP IV substrate GLP-1 was substituted by the amino acid analogue **1** to generate a novel analogue (**6**). To conserve the acidic charge of the P1' Glu residue, while maintaining the stereochemical arrangement of the tertiary-substituted β-carbon, a second GLP-1 analogue (**7**) was synthesized with the amino acid analogue **2** at the P1' position. The nomenclature and amino acid sequences of native and synthetic peptides examined in this work, as well as their corresponding P1'-modified analogues, are shown in Table 1. *In vitro* degradation assays were performed to determine the half-life of native GLP-1 and the P1'-modified GLP-1 analogues **6** and **7** in the presence of recombinant human DPP IV. As shown in Figure 3A, native GLP-1 was quickly hydrolyzed by DPP IV ($t_{½}$ ~ 0.3 h) (Table S1). Conversely, the analogues **6** and **7** remained intact over a 24 h incubation with the enzyme (Figure 3A).

Table 1. Amino acid sequences of native, synthetic, and P1'-modified protease substrates. Amino acid analogues are indicated by the numbers **1** (*tert*-Leu), **2** (β-dimethyl-Asp), and **3** (β-dimethyl-Gln).

Peptide/Analogue	Amino Acid Sequence
GLP-1	HA EGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH_2
6	HA **1**GTFTSDVSSYLEGQAAKEFIAWLVKGR-NH_2
7	HA **2**GTFTSDVSSYLEGQAAKEFIAWLVKGR-NH_2
8	HA **2**GTF**1**SDDSSY**1**EGQAAK**2**F**1**AW**1**VK**2**R-NH_2
GIP	YA EGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ-NH_2
9	YA **1**GTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ-NH_2
10	YA **2**GTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ-NH_2
OXM	HS QGTFTSDYSKYLDSRRAQDFVQWLMNTKRNRNNIA-NH_2
11	HS **1**GTFTSDYSKYLDSRRAQDFVQWLMNTKRNRNNIA-NH_2
12	HS **3**GTFTSDYSKYLDSRRAQDFVQWLMNTKRNRNNIA-NH_2
NPY	YP SKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-NH_2
13	YP **1**KPDNPGEDAPAEDMARYYSALRHYINLITRQRY-NH_2
14	YP **1**KPDNPGEDAPAEDMARYYSALRHYINLLTRPRY-NH_2
BNP	SP KMVQGSGCFGRKMDRISSSSGLGCKVLRRH-NH_2
15	SP **1**MVQGSGCFGRKMDRISSSSGLGCKVLRRH-NH_2
ENT	VP DPR-NH_2
16	VP **1**PR-NH_2
17	LLPR VA NGSSFVTV-OH
18	LLPRVA **1**GSSFVTV-OH
19	LLPR **1**ANGSSFVTV-OH

Secondary Structure. The secondary structure of GLP-1 and its analogues was examined by CD spectroscopy in the far UV region (185-260 nm). GLP-1, **6**, and **7** were dissolved in a sodium phosphate solution containing 30% trifluoroethanol (TFE). TFE induces and stabilizes helix and β-hairpin formation and mimics the membrane environment the peptide encounters just prior to binding its receptor.[29] As is characteristic of peptides and proteins with significant α-helical secondary structure the spectrum of native GLP-1 displayed minima at 222 and 208 nm

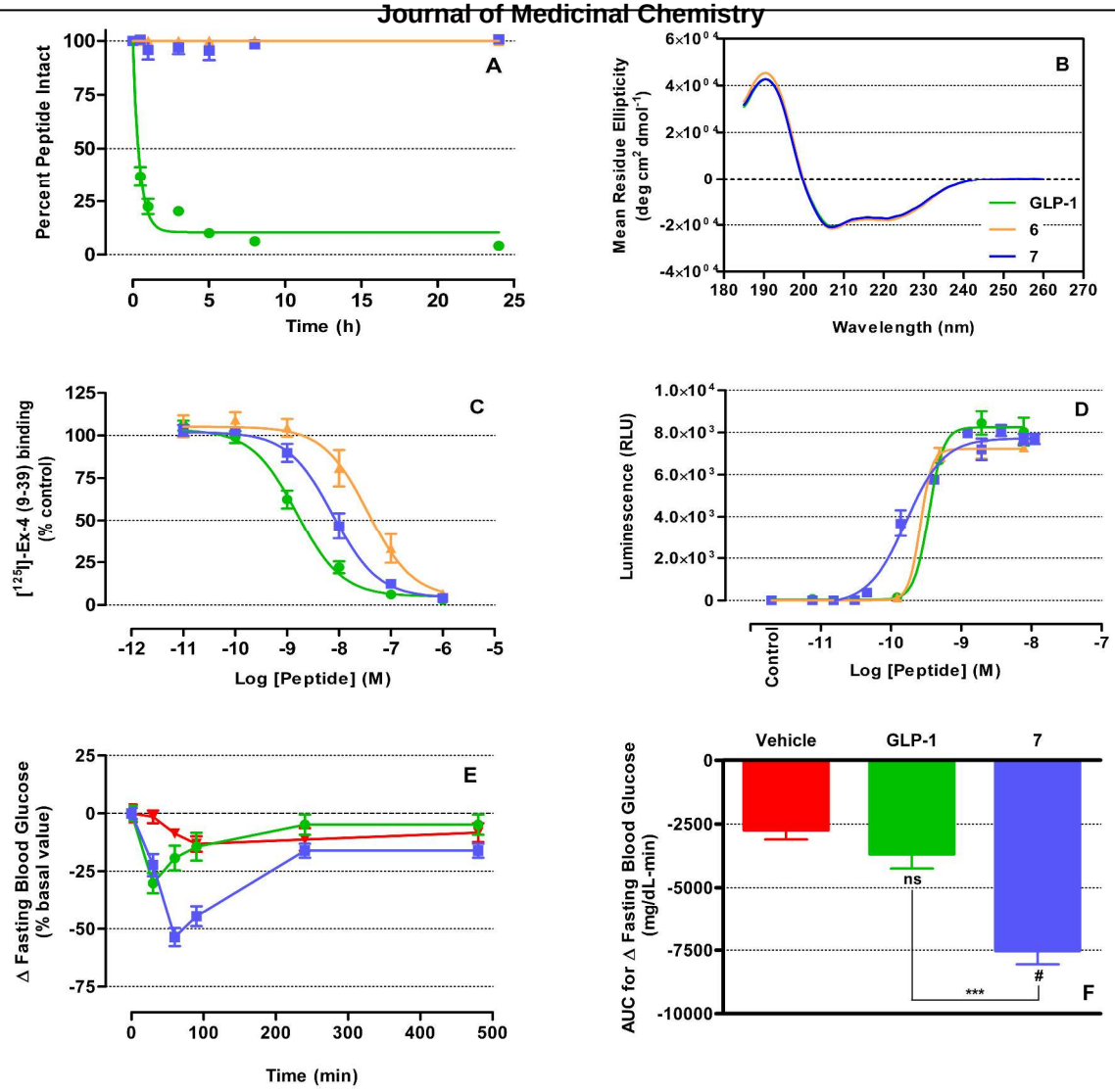

Figure 3. Characterization of P1'-modified GLP-1 analogues. (A) GLP-1 (●), **6** (▲), and **7** (■) were incubated with purified human DPP IV (5 nM) at 37 °C. The data represent the mean ± SEM of 3 independent experiments. (B) GLP-1, **6**, and **7** were dissolved in 10 mM sodium phosphate buffer containing 30% TFE and scanned from 260 to 185 nm on a CD spectrophotometer. The data represent the average of 4 scans. (C) The binding affinity of GLP-1 (●), **6** (▲), and **7** (■) was measured by [^{125}I]-Exendin-4 (9-39) competition binding experiments with COS-7 cells transiently expressing the human GLP-1R. The data represent the mean ± SEM of 3 independent experiments performed in duplicate. (D) GLP-1R activation by GLP-1 (●), **6** (▲), and **7** (■) was determined by measuring the stimulation of cAMP production. The data represent the mean ± range of 2 independent experiments performed in duplicate or triplicate. (E) Fasting blood glucose (FBG) measurements were determined prior to and at the indicated times after IP administration to *db*/*db* mice of vehicle (PBS) (▼) or 8 μg/mouse of GLP-1 (●) or **7** (■). The data represent the mean ± SEM percent change in FBG, compared to basal FBG levels, for 10 animals. (F) The change in FBG area under the curve (AUC) was calculated for each data set presented in panel E. The data represent the mean ± SEM change in FBG AUC (0 – 480 min) for 10 animals. Statistical significance: $^{\#}$, $p < 0.0001$ compared to vehicle; ns: non-significant compared to vehicle, ***, $p < 0.0001$ as indicated.

and a maximum at 190 nm (Figure 3B). The CD spectra of **6** and **7** were nearly identical to that of GLP-1 (Figure 3B), suggesting introduction of a tertiary-substituted β-carbon at the P1' position does not significantly change the secondary structure of native GLP-1.

Biological Activity. Competitive binding assays were performed with the radioligand [^{125}I]-Ex-4 (9-39) to determine the binding affinity of native GLP-1, **6**, and **7** at the human GLP-1R. As shown in Figure 3C, native GLP-1 bound the GLP-1R with high affinity to yield an IC_{50} value of 1.5 nM (Table S2). GLP-1's binding affinity was reduced to 36 nM when Glu^3 was replaced by the uncharged amino acid analogue **1** to generate analogue **6** (Figure 3C and Table S2). The binding affinity was restored to 7.6 nM when the charged amino acid analogue **2** was introduced at the P1' position to generate analogue **7** (Figure 3C and Table S2).

The *in vitro* biological activity of native and P1'-modified GLP-1 analogues at the human GLP-1R was determined by measuring cAMP production in response to receptor stimulation. As shown in Figure 3D, native GLP-1 and the P1'-modified GLP-1 analogues **6** and **7** activated the GLP-1R in a concentration-dependent manner. Introduction of the amino acid analogue **1** or **2** at the P1' position in GLP-1 had little if any impact on the peptide's potency at the GLP-1R (Figure 3D). The EC_{50} values obtained from fitted sigmoidal curves were 350, 260, and 170 pM for GLP-1, **6**, and **7**, respectively (Table S3).

The glucose-lowering efficacy of native GLP-1 and the P1'-modified GLP-1 analogue **7** was investigated in the *db*/*db* mouse model of T2D. Similar to human T2 diabetics, *db*/*db* mice display elevated fasting blood glucose (FBG) levels. The FBG levels of *db*/*db* mice were measured at various time points following ip administration of each peptide (8 μg/mouse). As shown in Figure 3E, thirty minutes after administration of native GLP-1 the average FBG level

of *db*/*db* mice was reduced by 30%. The glucose-lowering effect of native GLP-1 was, however, short-lived. Ninety minutes after administration, the FBG levels of *db*/*db* mice administered native GLP-1 returned to the levels observed in *db*/*db* mice administered vehicle (PBS) (Figure 3E). The P1'-modified GLP-1 analogue **7** elicited a more profound and long-lived glucose-lowering effect than native GLP-1. FBG levels of *db*/*db* mice were reduced by over 50% 60 min after administration of **7** and remained reduced by 45% for up to 90 min after peptide administration. At 240 min after administration, FBG levels had returned to basal levels in *db*/*db* mice receiving any of the three treatments (PBS, GLP-1, or **7**). The area under the FBG curve (0-480 min) for *db*/*db* mice administered either vehicle or 8 μg of peptide (GLP-1 or **7**) was calculated. In comparison to treatment with vehicle, treatment with **7** significantly ($p < 0.0001$) reduced the 8-h FBG AUC, whereas treatment with GLP-1 did not (Figure 3F). In addition, the 8-h FBG AUC was significantly ($p < 0.0001$) less in *db*/*db* mice treated with **7** than those treated with native GLP-1.

Safety. To evaluate the safety of extended treatment with P1'-modified peptides analogue **7** (1 mg/kg) was administered by ip injection to C57BL/6 mice once daily for seven days. The bodyweight of each mouse, as well as the food consumed per cage, was measured daily. Water consumption was measured on the third and seventh days of treatment. As shown in Figure S1, throughout the seven-day treatment, the bodyweight, food consumption, and water consumption of mice treated with analogue **7** was not statistically ($P > 0.05$) different than that of vehicle-treated animals.

On Day 7, following the final dose of vehicle or analogue **7**, necropsies were performed and the liver, kidneys, pancreas, heart, and brain were collected and weighed. No gross abnormalities

were noted in any of the twelve animals in the study. In addition, the organ weights of the vehicle-treated and peptide-treated animals were not statistically different ($p>0.05$) (Figure S1).

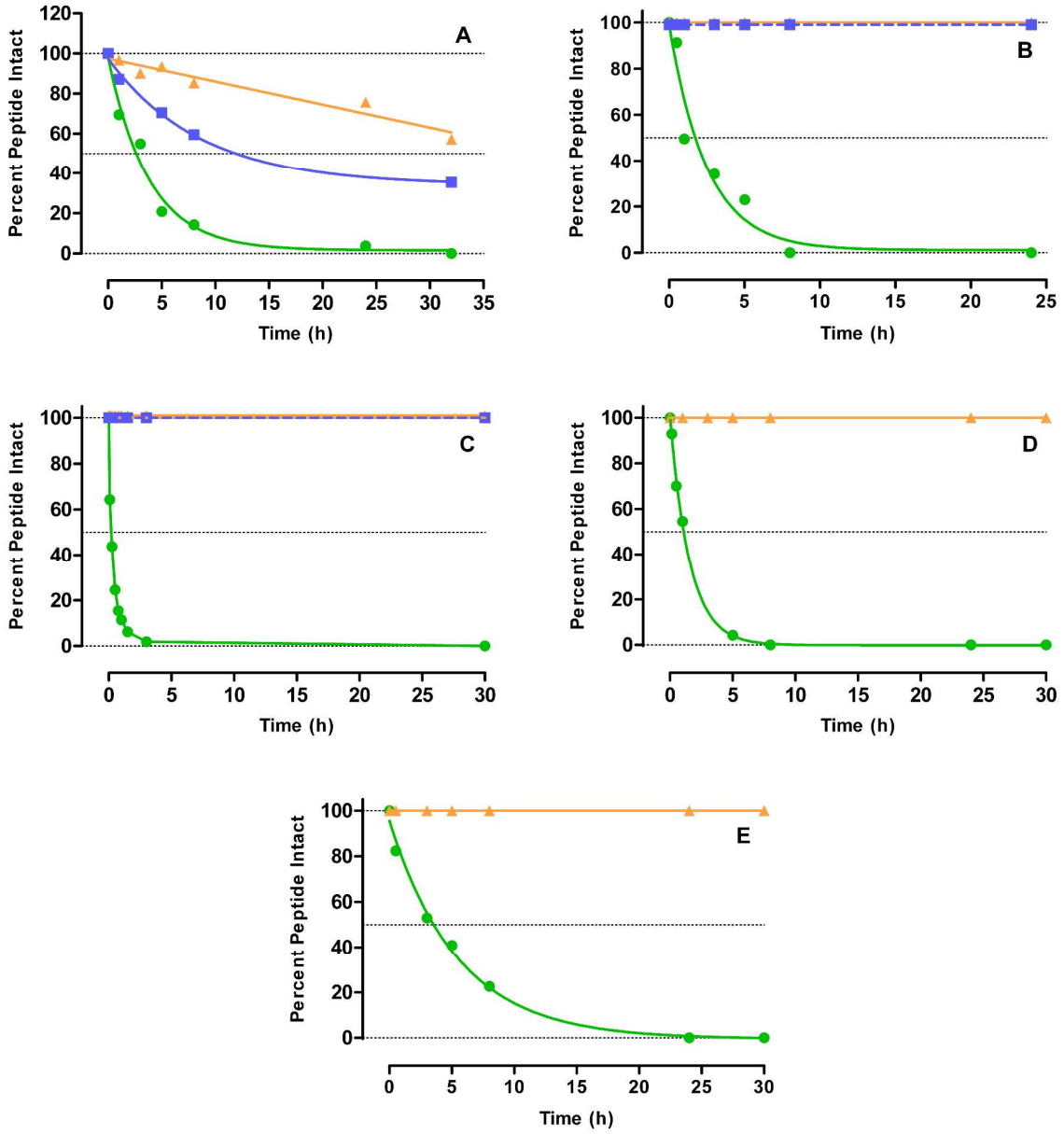

Figure 4. Degradation of native and P1'-modified DPP IV substrates. Peptides were incubated with purified human DPP IV at 37 °C. (A) GIP (●), **9** (▲), and **10** (■) were incubated with 10 nM DPP IV. (B) OXM (●), **11** (▲), and **12** (■) were incubated with 50 nM DPP IV. (C) NPY (●), **13** (▲), and **14** (■) were incubated with 5.0 nM DPP IV. (D) BNP (●) and **15** (▲) were incubated with 0.5 nM DPP IV. (E) ENT (●) and **16** (▲) were incubated with 10 nM DPP IV. The data in panels A-E represent a single experiment.

Protease-resistant Analogues of Other DPP IV Substrates. *In Vitro Degradation*. The endogenous DPP IV substrates GIP, OXM, NPY, BNP, and ENT were synthesized with the amino acid analogue **1** at the P1' position to generate the analogues **9**, **11**, **13**, **15**, and **16**,

respectively (Table 1). Take note that the carboxylic acid normally found at the C-terminus of native GIP, OXM, BNP, and ENT was replaced by an amide in "native" and "P1'-modified" peptides to prevent degradation by carboxypeptidases in future *in vivo* studies. In addition, to demonstrate the applicability of the method to receptor-selective agonists, an analogue (**14**) of the Y1R-selective peptide [$Leu^{31}Pro^{34}$]NPY was synthesized with **1** at the P1' position (Table 1). A GIP analogue (**10**) containing the amino acid analogue **2** at the P1' position was synthesized so as to conserve the acidic charge of the P1' Asp residue present in native GIP (Table 1). Likewise, an OXM analogue (**12**) containing the amino acid analogue **3** at the P1' position was synthesized so as to conserve the basic charge of the P1' Gln residue present in native OXM (Table 1). *In vitro* degradation assays revealed that the P1'-modified analogues **11**, **12**, **13**, **14**, **15**, and **16** were completely resistant to DPP IV-mediated degradation for 24 h or more (Figure 4). The P1'-modified GIP analogues **9** and **10** were degraded by DPP IV, but at a significantly slower rate than native GIP ($t_{½}$ ~ 3 h) (Figure 4A and Table S1). The half-lives of the analogues **9** and **10** were estimated to be > 30 h and 10 h, respectively. The half-lives of native GLP-1, GIP, OXM, NPY, BNP, ENT and their respective P1'-modified analogues in the presence of DPP IV are provided in Table S1.

Secondary Structure. The secondary structure of native GIP, OXM, and NPY and their P1'-modified analogues **9**, **10**, **11**, **12**, **13**, and **14** was examined by CD spectroscopy in the far UV region (185-260 nm) as described above for native GLP-1, **6**, and **7**. Similar to the results obtained for GLP-1 and its P1'-modified analogues, the CD spectra of each native peptide and its respective P1'-modified analogue(s) were nearly identical (Figure S2).

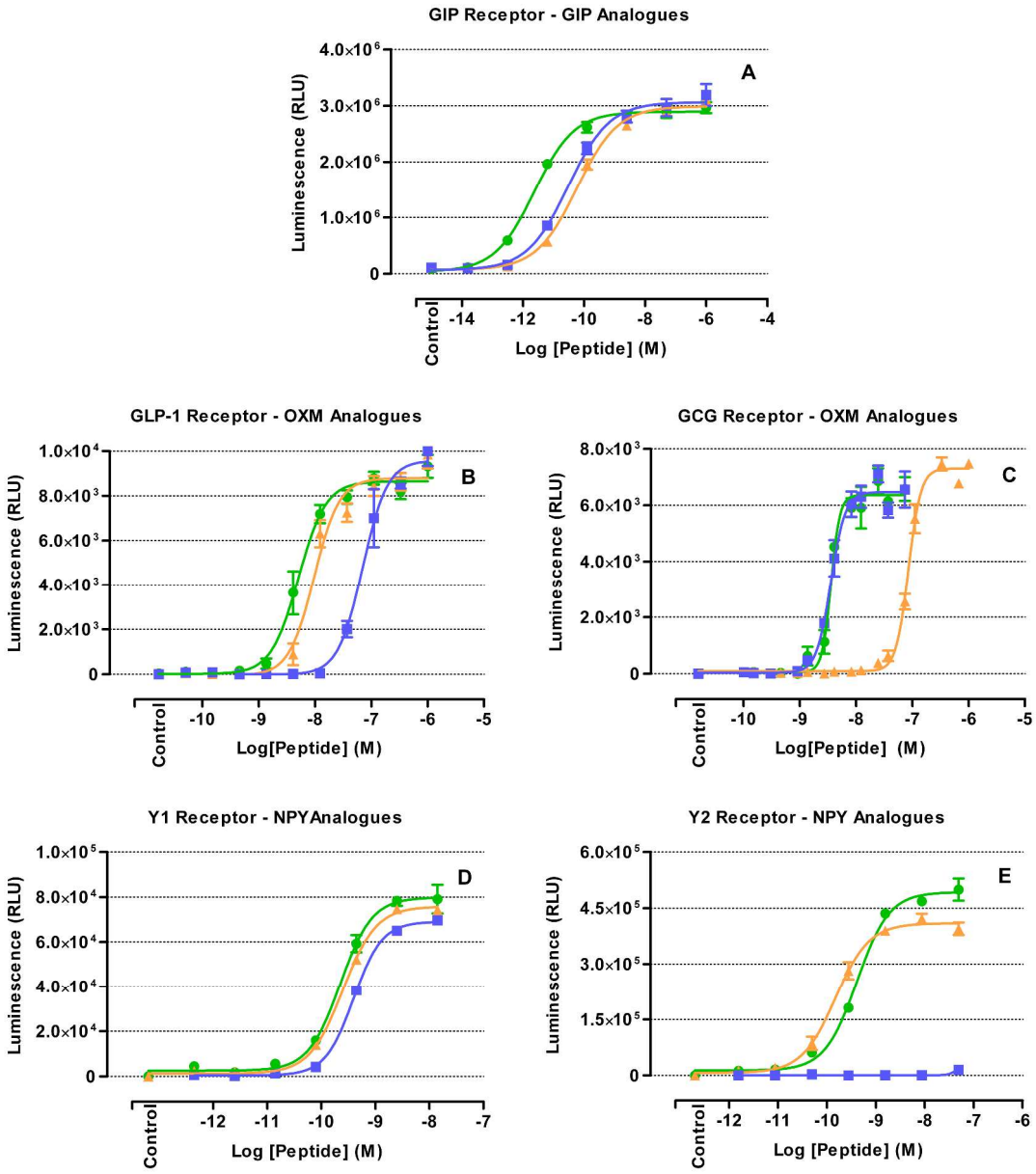

Figure 5. Concentration-response curves for GIP, GLP-1, GCG, Y1, and Y2 receptor activation. Half-maximal effective concentration (EC_{50}) values were determined by measuring activation of a cAMP-dependent reporter gene (panel A), stimulation of cAMP production (panels B and C), or recruitment of β-arrestin to the receptor (panels D and E). (A) Agonist activity of GIP (●), **9** (▲), and **10** (■) at the GIPR. Agonist activity of OXM (●), **11** (▲), and **12** (■) at the GLP-1R (B) or GCGR (C). Agonist activity of NPY (●), **13** (▲), and **14** (■) at the Y1R (D) or Y2R (E). The data represent the mean ± SEM of two independent experiments performed in duplicate or triplicate.

Biological Activity. The *in vitro* biological activity of native and P1'-modified forms of GIP, OXM, and NPY were determined at their respective receptor(s) by measuring activation of a cAMP-dependent reporter gene (Figure 5A), stimulation of cAMP production (Figure 5B and 5C), or β-arrestin recruitment to the receptor (Figure 5D and 5E). As shown in Figure 5, all native and P1'-modified peptides activated their respective receptors in a concentration-dependent manner. Introduction of the amino acid analogue **1** or **2** at the P1' position in GIP generated the analogues **9** and **10** and decreased the peptide's potency at the GIPR by approximately 20- or 10-fold, respectively (Figure 5A). As previously reported, native OXM acted as a dual agonist at the GLP-1 and glucagon (GCG) receptors (Figure 5B and 5C). Introduction of the amino acid analogue **1** at the P1' position in OXM, to generate analogue **11**, reduced the potency of the peptide at the GLP-1R and CGCR by approximately 2- and 20-fold, respectively (Figure 5B and 5C). Introduction of the amino acid analogue **3** at the P1' position in OXM, to generate analogue **12**, did not change the peptide's potency at the GCGR, but decreased the peptide's potency at the GLP-1R by more than 10-fold (Figures 5B and 5C). The NPY analogues **13** and **14**, both of which have the amino acid analogue **1** at the P1' position, retained agonist activity similar to that of native NPY at the Y1R (Figure 5D). The NPY analogue **13** also potently activated the Y2R, while the Y1R-selective analogue **14** did not, even at concentrations in the high nanomolar range (Figure 5E). Corresponding EC_{50} values for native and P1'-modified analogues are provided in Table S3.

Application of the P1' Modification to Other Enzyme Substrates. *DPP IV Homologs: FAPα and DPP8*. The P1'-modified NPY analogues **13** and **14** were exposed to the DPP IV homologs FAPα and DPP8 (Figure 6). Similar to DPP IV, these enzymes cleave on the carboxyl side of the Pro^2 residue in NPY to generate the truncated metabolite NPY_{3-36}.[30, 31] In the presence of

FAPα the half-life of native NPY was 4.8 h, whereas the analogues **13** and **14** had half-lives of greater than 50 h, with approximately 70% of both remaining intact after 48 h (Figure 6B and Table S1). Interestingly, less than 50% of native NPY was digested after an 8 h exposure to DPP8 (Figure 6A). The P1'-modified analogue **13**, on the other hand, remained completely intact over an 8 h incubation with DPP8 (Figure 6A).

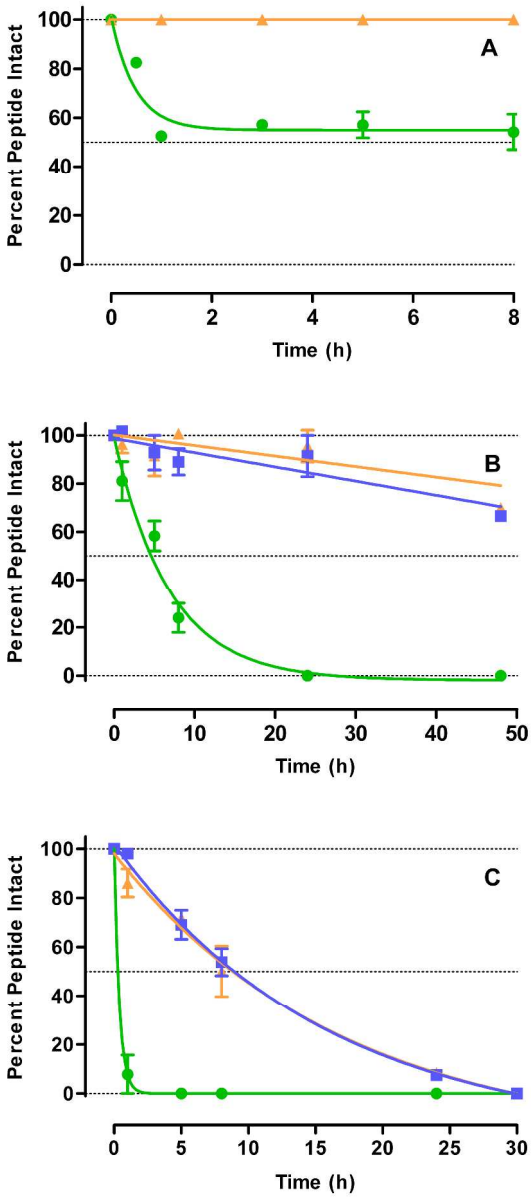

Figure 6. Degradation of NPY and P1'-modified analogues by DPP8, FAPα, or APP2. NPY (●), **13** (▲), and **14** (■) were incubated with purified (A) DPP8 (100 nM), (B) FAPα (25 nM), or (C) APP2 (100 nM) at 37 °C. The data represent the mean ± range of two (A and C) or mean ± SEM of three (B) independent experiments.

Metalloprotease: APP2. NPY is also metabolized by the metalloprotease APP2, which cleaves after the N-terminal Tyr[1] residue to generate $NPY_{2\text{-}36}$.[32] In the presence of APP2, native NPY had a half-life of approximately 0.3 h and was almost completely degraded to $NPY_{2\text{-}36}$ after a 1 h incubation (Figure 6C and Table S1). Under the same conditions, the P1'-modified analogues **13** and **14** had greatly extended half-lives of 11.7 and 11.0 h, respectively (Figure 6C and Table S1).

Serine Proteases: Trypsin, Chymotrypsin, αLP. A synthetic endoprotease substrate (**17**) was designed in our laboratory to contain both a post-

Ala cleavage site for the archetypal serine protease αLP and a post-Arg cleavage site for trypsin (Table 1). The substrate analogues **18** and **19** were generated by replacing the P1' residue at the cleavage sites for αLP and trypsin, respectively, in **17** with the amino acid analogue **1** (Figure 7 and Table 1). In the presence of purified αLP or trypsin the unmodified endoprotease substrate **17** had a half-life of approximately 0.4 h (Figure 7 and Table S1). Conversely, the P1'-modified analogues **18** and **19** remained completely intact when incubated with αLP or trypsin for 5 or 8 h, respectively (Figure 7).

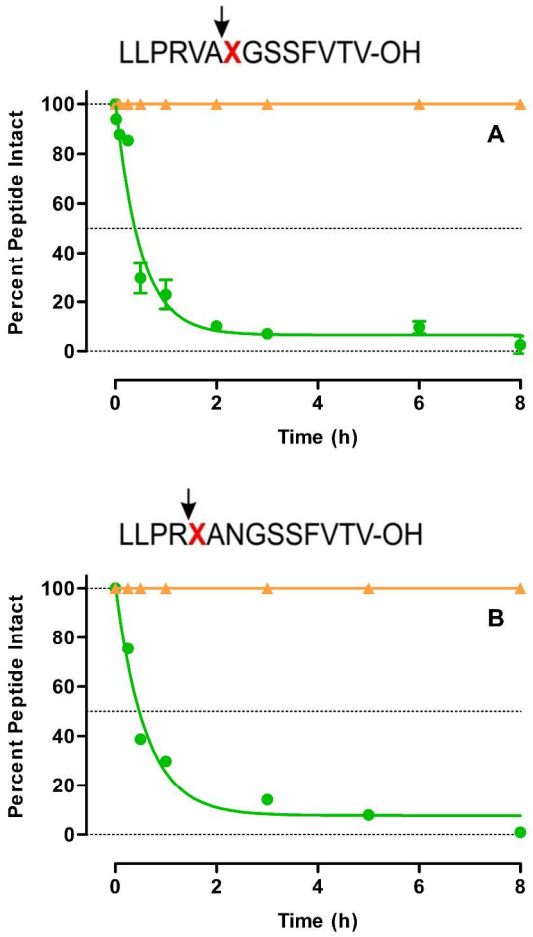

Figure 7. Degradation of unmodified and P1'-modified substrates of αLP and trypsin. (A) **17** (●) and **18** (▲) were incubated with purified αLP (10 nM) at 37 °C. (B) **17** (●) and **19** (▲) were incubated with purified trypsin (10 nM) at 37 °C. Sites of αLP (A) and trypsin (B) cleavage are shown. The data represent a single experiment.

To further demonstrate the widespread applicability of this method, a GLP-1 analogue (**8**) containing the amino acid analogues **1** or **2** at the P1' position of trypsin and chymotrypsin cleavage sites was also generated. The stability of the multiply-modified GLP-1 analogue **8** was investigated in the presence of the digestive enzymes trypsin and chymotrypsin (Figure 8). Unmodified native GLP-1 was completely degraded after an incubation of only 10 s with either trypsin or chymotrypsin (Figure 8). In contrast, **8** remained completely intact over a 5 h incubation with

trypsin (Figure 8A). The rate of degradation of **8** was significantly reduced in comparison to that of native GLP-1 in the presence of chymotrypsin, with 50% of **8** remaining intact after ~ 0.6 h (Figure 8B and Table S1).

Kinetic Analysis of Peptide-DPP IV Interaction. It is possible that P1'-modified peptides are less susceptible to degradation by DPP IV because (i) the modified substrates lose affinity for the enzyme, or (ii) binding to the active-site is conserved but subsequent catalytic cleavage is compromised. Kinetic analyses were performed to explore these options and determine whether and to what extent P1'-modified substrates inhibit DPP IV activity. In brief, the initial rate of DPP IV-mediated cleavage of the chromogenic substrate Ala-Pro-*p*ara-nitroanilide (AP-*p*NA) was measured in the presence of increasing concentrations of the P1'-modified analogues **9**, **13**, **14**, **15**, or **16**. Modified peptides were found to competitively inhibit DPP IV, with K_i values of 160 ± 70 for **9**, 6.3 ± 0.8 for **13**, 5.0 ± 0.6 for **14**, and 61 ± 7 μM for **15** (Figure S3 and Table S4). These values are in the range of reported K_m values of DPP IV for the corresponding unmodified

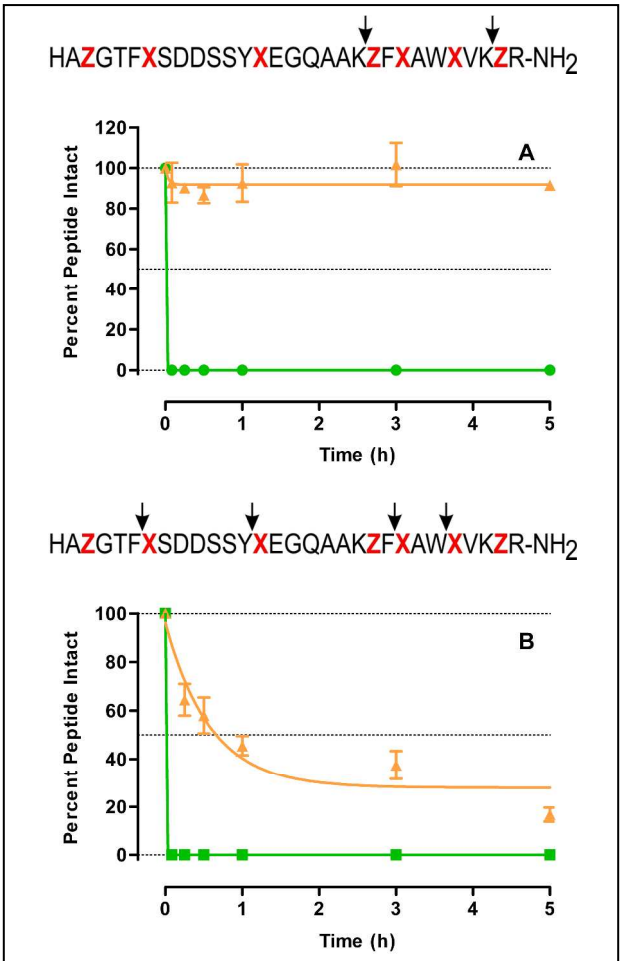

Figure 8. Degradation of GLP-1 and **8** by trypsin and chymotrypsin. GLP-1 (●) and **8** (▲) were incubated with purified (A) trypsin (100 nM) or (B) chymotrypsin (100 nM) at 37 °C. Predicted sites of trypsin (A) and chymotrypsin (B) cleavage within the sequence of **8** are shown. The data represent the mean ± range of two independent experiments.

peptides (Table S4).[33-35] Although to the best of our knowledge the K_m of ENT with DPP IV has not been reported, the low K_i (4.5 ± 0.7 μM) value of the P1'-modified ENT analogue (**16**) determined in our experiments implies that the P1'-modified analogue binds with a relatively high affinity to the DPP IV active-site (Figure S3E). Thus, it appears that while the enzyme affinity of modified peptides is largely conserved, their catalytic cleavage is compromised. As expected, native peptides had markedly higher K_i values for inhibition of DPP IV than their P1'-modified counterparts. For example, the K_i for inhibition of DPP IV by ENT was 142 ± 43 μM, while the K_i for inhibition of DPP IV by **16** was only 4.5 ± 0.7 μM (Table S4). This is likely due to the fact that the native peptides are rapidly degraded by DPP IV and the resulting metabolites do not act as competitive inhibitors of the enzyme.

DISCUSSION

The use of peptides as therapeutic agents is greatly limited by their susceptibility *in vivo* to degradation and inactivation by a wide range of proteases. In this study we describe a simple and general method of generating metabolically stable peptide analogues that retain potent biological activity. The method involves substituting the P1' residue, which is positioned directly C-terminal of the protease cleavage site, with an amino acid analogue containing a tertiary-substituted β-carbon (ie. **1**, **2**, or **3**). Application of this method to the endogenous DPP IV substrates GLP-1, GIP, OXM, NPY, BNP, and ENT generated novel P1'-modified peptide analogues that were highly resistant to DPP IV-mediated degradation.

The influence of the P1' residue on susceptibility to DPP IV-mediated cleavage has been previously examined with GLP-1, GIP, and OXM. Green and colleagues found that replacing the P1' Glu residue in GLP-1 with a Pro or Lys residue greatly enhanced the half-life of GLP-1 in the presence of DPP IV, while replacing the P1' residue with a Tyr or Phe residue only moderately enhanced the half-life.[36, 37] An investigation of the stability of Glu^3-substituted GIP analogues in the presence of DPP IV revealed GIP analogues containing an Ala, Phe, Trp, or Tyr residue at the P1' position were quickly degraded by DPP IV, yielding half-lives similar to that of native GIP.[38] Substitution of Glu^3 with a Lys residue slightly enhanced the stability of GIP in the presence of DPP IV, but only substitution with a Pro residue completely blocked DPP IV-mediated degradation of GIP.[38] Substitution of the Gln^3 residue in OXM with a Glu residue, in combination with other N-terminal substitutions (ie. D-His^1 or Ala^2), rendered the peptide resistant to DPP IV-mediated degradation over a 2 h incubation.[39] When the Gln^3 residue in OXM was substituted with an Asp, Glu, Leu, noroleucine (Nle), or Pro residue, only the OXM analogue containing an Asp residue at the P1' position displayed significant resistance to DPP

IV-mediated degradation.[40] To our knowledge, the influence of amino acid substitutions at the P1' position in NPY, BNP, or ENT on DPP IV-mediated degradation has never been investigated and this is the first report of a DPP IV-resistant NPY, BNP, or ENT analogue.

CD studies revealed that introduction of the amino acid analogues **1**, **2**, and/or **3** at the P1' position in GLP-1, GIP, OXM, and NPY did not significantly alter the secondary structure of the peptides, which is required to maintain high affinity and potent activity at their respective receptors (Figure S2).[41-44] The effect of P1' residue substitutions on agonist activity has been previously investigated for GLP-1, GIP, and OXM. GLP-1 analogues containing an Ala or Asp residue at the P1' position were 30-fold less potent and considerably less active than native GLP-1.[45] Replacement of the Glu^3 residue in GLP-1 with a Pro, Phe, or Tyr residue also reduced binding affinity and agonist activity at the GLP-1R, and an analogue containing a basic Lys residue at the P1' position even acted as an antagonist at the GLP-1R.[36, 37] Conversely, in our study, replacement of the P1' residue in GLP-1 with the amino acid analogue **1** or **2** did not significantly alter the peptide's binding affinity or agonist activity at the GLP-1R. In fact, the P1'-modified analogue **7** was ~2-fold more potent than native GLP-1 at the GLP-1R and demonstrated more potent and long-lived glucose-lowering activity than the native peptide in diabetic mice. Furthermore, daily administration of analogue **7** to lean mice for seven days did not cause any gross abnormalities or significant changes in body weight, organ (liver, pancreas, kidneys, heart, brain) weight, food consumption, or water consumption.

Site-directed mutagenesis studies of the GIPR have suggested that the Glu^3 residue in GIP interacts directly and uniquely with residue Arg^{183} in the GIPR to stimulate cAMP production.[46] GIP analogues containing an Ala, Trp, or Tyr residue at the P1' position were reported to display slightly reduced activity at the GIPR (2- to 3-fold) and caused notable inhibition (12-24%) of

GIP-stimulated cAMP production.[38] Replacement of Glu^3 in GIP with a Lys, Phe, Pro, or Hydroxyproline (Hyp) residue produced potent GIPR antagonists.[38, 47] In our study, replacement of the P1' Glu^3 residue with the amino acid analogue **1** or **2** moderately reduced the potency of GIP at the GIPR by 20- or 10-fold, respectively. We suggest the GIP analogue **10** is a slightly more potent GIPR agonist than analogue **9** because the amino acid analogue **2** in **10** conserves the acidic charge of the Glu^3 residue present in native GIP. The reduced potency of **10** in comparison to native GIP may be due to a difference in how the carboxylate group of Asp in **10** and Glu in native GIP is positioned to interact with residues in the GIPR. We propose that a GIP analogue containing a β-dimethyl-Glu residue at the P1' position may be more equivalent in potency to native GIP than either **9** or **10**.

OXM is a dual agonist with activity at both the GLP-1R and the GCGR.[48-50] Several studies have demonstrated that the Gln^3 residue in OXM is essential for GCGR recognition and activation. In comparison to native OXM, analogues containing a Glu, Asp, Asn, Leu, or noroleucine (Nle) residue at the P1' position displayed greatly reduced activity at the GCGR, but retained potent activity at the GLP-1R.[40] In agreement with previously published studies, we found that a basic charge at the P1' position in OXM is necessary for the peptide to retain activity comparable to native OXM at the GCGR. Introduction of the uncharged amino acid analogue **1** at the P1' position in OXM significantly reduced the peptide's potency at the GCGR, but not at the GLP-1R. Conversely, introduction of the basic amino acid analogue **3** at the P1' position significantly reduced activity at the GLP-1R, but not at the GCGR. Loss of GLP-1R activity with the latter peptide derivative was surprising, since native OXM, which contains a basic Gln residue at the P1' position, is a potent activator of the GLP-1R.

It is well-established that both an intact N- and C-terminus are required for NPY to bind the Y1R subtype.[43, 51] In fact, the binding affinity of NPY to the Y1R is decreased by 100-fold or >1000-fold, respectively, when the N-terminal Tyr amino acid residue or Tyr-Pro dipeptide is removed.[43] In contrast, an intact N-terminus is not required for NPY to bind and activate the Y2R subtype.[52] The P1'-modified analogue **13** retained potent agonist activity at both the Y1R and Y2R. Our P1'-modified derivative of the Y1R-selective NPY analogue, referred to as analogue **14**, acted as a potent agonist at the Y1R, but, as expected, displayed no activity at the Y2R.

The presence of the amino acid analogue **1** at the P1' position in NPY also rendered the analogues **13** and **14** resistant to cleavage by the DPP IV homologs DPP8 and FAPα and the metalloprotease APP2. Interestingly, less than 50% of native NPY was digested after an 8 h exposure to DPP8. A recently published study suggests DPP IV-generated metabolites can bind to a secondary substrate binding site on the enzyme, outside the S2-S2' region, to inhibit subsequent enzyme activity.[53] Perhaps the DPP8-generated metabolite NPY_{3-36} binds to a secondary binding site on DPP IV to inhibit cleavage of the remaining full-length NPY. In the NPY analogues **13** and **14** the P2' position of the APP2 cleavage site is occupied by the bulky amino acid analogue **1**. The fact that APP2 is reported to disfavor P2' residues with bulky side-chains[54] may explain its inability to degrade analogues **13** and **14**.

Having demonstrated that the P1' modification protected substrates of several amino/dipeptidases from degradation, we explored whether this method of modification could also prevent the degradation of endoprotease substrates. We found that a synthetic peptide substrate containing the amino acid analogue **1** at the P1' position of the αLP and trypsin cleavage sites was resistant to αLP- and trypsin-mediated degradation, respectively. In addition,

introduction of the amino acid analogues **1** or **2** at the P1' position of the trypsin and chymotrypsin cleavage sites in GLP-1 greatly extended the peptide's half-life in the presence of these digestive enzymes.

Kinetic analyses were performed to investigate the mechanism by which the P1' modification renders peptides resistant to DPP IV-mediated degradation. Kinetic analyses suggested that the P1'-modified substrates (ie. **9**, **13**, **14**, **15**, and **16**) act as competitive inhibitors of DPP IV (Figure S3). The efficacy of P1'-modified analogues as competitive inhibitors of DPP IV varied, with corresponding K_i's ranging from low to high micromolar values (Table S4). Due to the presence of conformationally restrained amino acid residues (ie. **1**, **2**, or **3**) at the P1' position, it is likely that modified peptides analogues bind within the active-site of the enzyme in a constrained conformation. We propose that in this conformation the peptide has enough flexibility to establish the interactions necessary to retain high binding affinity at the enzyme active-site. The peptide conformation may be too restricted, however, to establish the interactions necessary for complete hydrolysis of the scissile bond by DPP IV. In modeling studies the hexapeptide NPY_{1-6} was found to be positioned similarly within the active-sites of DPP IV and FAPα.[55] Therefore, it is likely that the P1' modification confers resistance to FAPα- and DPP IV-mediated degradation by a similar mechanism. It may be necessary to obtain the crystal structure of a P1'-modified analogue in the active-site of DPP IV in order to elucidate the exact mechanism by which the P1' modification renders peptides resistant to enzymatic degradation.

Conclusion

More than 60 biologically active hormones, growth factors, chemokines, and neuropeptides have been identified as physiological substrates of DPP IV. The results presented herein suggest that any of these substrates could be made resistant to DPP IV-mediated degradation by introduction of the P1' modification. Moreover, the proposed method works when applied to peptide substrates of other proteases, such as FAPα, trypsin, and chymotrypsin. It should be possible to synthesize and substitute a tertiary-substituted β-carbon analogue for almost every naturally occurring amino acid -- a general platform for enhancing the enzymatic stability of peptide-based therapeutics.

EXPERIMENTAL SECTION

Peptide Synthesis. The following peptides were synthesized and purified by the Tufts University Core Facility (TUCF): NH_2-Ala-Pro-Leu-Ser-Trp-Ser, NH_2-Ala-Pro-Ile-Ser-Trp-Ser, NH_2-Ala-Pro-Tle-Ser-Trp-Ser, GLP-1, OXM, NPY, ENT, **6**, **7**, **8**, **11**, **13**, **14**, **16**, **17**, **18**, and **19**. Native GIP and BNP, and the modified peptides **9**, **10**, **12**, and **15** were synthesized and purified by CS Bio (Menlo Park, CA). The purity (> 95%) of all peptides was confirmed by HPLC-MS. The modified amino acid Fmoc-L-*tert*-Leu was purchased from Novabiochem. N-Fmoc-L-β, β-dimethyl-Asp(*t*-butyl) (**4**) and N-Fmoc-L-β, β-dimethyl-Gln-Gly(*t*-butyl)-OH (**5**) were synthesized in-house (Supporting Information).

***In Vitro* Degradation Assays.** In vitro degradation assays were performed to determine the stability of native and modified peptides in the presence of various enzymes. For all degradation assays peptides (0.1 mM) were incubated at 37 °C with purified enzyme. At specified times, samples were collected from the degradation reactions and enzymatic activity was terminated with the addition of 10% (v/v) 0.1 M HCl.

Synthetic hexapeptides were incubated in DPP IV assay buffer (50 mM HEPES, 0.14 M NaCl, pH 8.0) with 10 mU/mL DPP IV purified from porcine kidney (Sigma-Aldrich, USA). Native and P1'-mofidied analogues of GLP-1, GIP, OXM, NPY, BNP, and ENT were incubated in DPP IV assay buffer with purified recombinant human DPP IV (R&D Systems) at the concentrations indicated in the corresponding Figure. Native and P1'-modified NPY analogues were incubated in DPP IV assay buffer with 25 nM purified recombinant human FAPα (R&D Systems) or 100 nM purified recombinant human DPP8 (BPS Biosciences). Additionally, native and P1'-

modified analogues of NPY were incubated in APP2 assay buffer (100 mM HEPES, 500 mM MnCl2, pH 8.0) with 100 nM purified recombinant human APP2 (R&D Systems).

The unmodified (**17**) and P1'-modified synthetic substrate (**18**) of the enzyme αLP were incubated in αLP assay buffer (50 mM Tris-HCl, 100 mM KCl, pH 8.75) with 10 nM αLP purified from *Lysobacter enzymogenes*.[56] Similarly, the unmodified (**17**) and P1'-modified synthetic substrate (**19**) of the enzyme trypsin were incubated in trypsin assay buffer (50 mM Tris-HCl, 20 mM $CaCl_2$, pH 8.0) with 10 nM purified porcine pancreatic trypsin (Sigma, USA). Native GLP-1 and **8** were incubated with 100 nM purified porcine pancreatic trypsin in trypsin assay buffer, or with 10 nM purified bovine pancreatic chymotrypsin (Sigma, USA) in chymotrypsin assay buffer (100 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.0).

Samples were analyzed by reverse-phase HPLC-MS on a Thermo Finnigan LCQ Duo instrument equipped with a Zorbax C-18 ECLYPSE column (2.2 x 50 mm, 3.5 μm) and a UV detector. With the exception of ENT digests, peptide fragments were separated by linear gradient elution at a flow rate of 0.2 mL/min beginning at 80% solvent A/20% solvent B and shifting to 30% solvent A/70% solvent B over 20 min, where solvent A is water (0.1% TFA) and solvent B is acetonitrile (0.08% TFA). The eluents were monitored by UV absorption and mass spectrometry. ENT peptide fragments were separated by linear gradient elution beginning at 100% solvent A/0% solvent B and shifting to 50% solvent A/50% solvent B. The amount of intact peptide was quantified by integrating the corresponding area under the curve in each extracted ion chromatogram. This value was expressed as "Percent Peptide Intact", relative to the corresponding value before exposure to enzyme digestion (time 0). The data were fit to a single-phase exponential equation in GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA) to determine half-life values.

Circular Dichroism. Peptides were dissolved to a nominal concentration of 30 μM (as determined by UV absorbance) in 10 mM sodium phosphate buffer (pH 7.0) containing 30% TFE. Actual peptide concentrations were calculated by measuring the UV absorbance at 280 nm and using a molar extinction coefficient of 6970 $M^{-1}cm^{-1}$ for GLP-1 and its analogues, 13980 $M^{-1}cm^{-1}$ for GIP and its analogues, 8250 $M^{-1}cm^{-1}$ for OXM and its analogues, and 7450 $M^{-1}cm^{-1}$ for NPY and its analogues. The concentration of BNP, which lacks any aromatic amino acid residues, was determined by BCA assay (Thermo Scientific, USA). Far-UV spectra were collected on a Jasco J-810 circular dichroism spectropolarimeter (Jasco Inc., Easton, MD) at 25 °C in a 1 mm quartz cuvette. Four scans of spectral data were accumulated from 260 to 190 nm with a response time of 2 s, and from 185 to 200 nm with a response time of 16 s. In all cases, a bandwith of 1 nm, data pitch of 0.5 nm, and scanning speed of 10 nm/min were employed. For data analysis, the solvent signal was subtracted and millidegree values were converted to mean residue ellipticity (deg cm^2 $dmol^{-1}$) using the following formula:

$$[\Theta]_{MRW} = (\Theta_{obs} \cdot 100 \cdot M_r) / (c \cdot l \cdot N_A);$$

where $[\Theta]_{MRW}$ is mean residue ellipticity, Θ_{obs} is observed ellipticity in degrees, M_r is the peptide's molecular weight, c is the peptide concentration in mg/mL, l is the pathlength in cm, and N_A is the number of amino acids in the peptide. GraphPad Prism 5 software was used to fit and smooth curves and to graph mean residue ellipticity as a function of wavelength.

Receptor Binding Assay. To determine the binding affinity of native and modified GLP-1 analogues at the GLP-1R competitive binding experiments were performed as previously described.[57] Briefly, COS-7 cells transiently expressing the GLP-1R were treated with peptides together with 17 pM of the radioligand [^{125}I]-exendin(9-39). Positive control wells contained the

radioligand in the absence of competing ligand. Nonspecific binding of the radioligand was determined in the presence of 1 μM unlabeled exendin(9-39). The amount of cell-bound radioactivity was determined by liquid scintillation counting (Beckman Gamma counter 5500B). Data were normalized to [^{125}I]-exendin(9-39) binding in the absence of competitor and fit to the one-site competition model in GraphPad Prism 5 to determine half-maximal binding affinity (IC_{50}) values.

Receptor Activation Assays. The agonist activity of native and modified GLP-1 analogues at the GLP-R and native and modified OXM analogues at the GLP-1R and GCGR was determined using cAMP Hunter eXpress GPCR Assay kits (DiscoveRx, USA) according to the manufacturer's protocol. The agonist activity of native and modified NPY analogues at the Y1 and Y2 receptors was determined using PathHunter eXpress β-arrestin kits (DiscoveRx, USA) according to the manufacturer's protocol. Luminescence levels were measured using a Perkin Elmer Victor^{3}V plate reader (Waltham, MA) and corrected for with luminescence levels from untreated control wells. Sigmoidal concentration-response variable slopes were fit to the corrected data in GraphPad Prism 5 and EC_{50} values were determined.

The agonist activity of native and modified GIP analogues at the GIPR was determined by use of a previously described luciferase reporter gene assay (Fortin, 2010). In brief, HEK293 cells transiently transfected with cDNAs encoding β-galactosidase (β-gal) under the control of a constitutively active CMV promoter, a cAMP responsive element binding-driven firefly luciferase reporter gene, and the human GIP receptor (or a vector control) were stimulated for 6 h with vehicle (serum-free medium) or varying concentrations of GIP, sGIP, or bGIP. Luciferase activity was measured by use of Steadylite reagent (PerkinElmer Life and Analytical Sciences, Waltham, MA) with a PerkinElmer Victor X3 plate reader. β-gal activity was used to

correct the corresponding luciferase data for interwell variability in transfection efficiency and/or cell survival. Data normalized to vehicle-treated controls were fit to sigmoidal concentration-response curves in GraphPad Prism 5 and EC_{50} values were determined.

Safety Study. Six male and six female seven-week-old C57BL/6 mice were purchased from Charles River Laboratory (Cambridge, MA) and housed in same-sex cages consisting of three mice per cage with a 12 hour light:12 hour dark cycle. Following a twelve day acclimation period mice were randomly assigned into two groups (vehicle and experimental), each consisting of one cage of male mice (n = 3) and one cage of female mice (n = 3). Mice in the vehicle group were administered PBS (pH 7.4) while mice in the experimental group were administered analogue **7** (1 mg/kg) dissolved in PBS. Treatments were administered once daily (9 - 11 am) by ip injection for seven days. Just prior to dosing, body weight was recorded for each mouse and food consumption was recorded for each cage. Water consumption was recorded on the third and seventh days of the experiment. On the last day of the experiment (Day 7), following the final dose, the animals were euthanized and necropsies were performed. The liver, pancreas, kidneys, heart, and brain of each animal were collected and weighed.

***In Vivo* Glucose-lowering Activity.** Nine-week-old diabetic BKS.Cg m +/+ Leprdb/J (*db*/*db*) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed two to three per cage with a 12 hour light:12 hour dark cycle. Following an overnight fast, lightly anesthetized (3% isofluorane) mice were administered an 8 μg (approximately 0.2 mg/kg) dose of peptide dissolved in PBS by ip injection. Control mice were treated with 0.4 mL vehicle (PBS) instead of peptide. Blood samples were collected from the tail vein of conscious mice at 0.5, 1.0, 1.5, 4, or 8 h after injection and FBG levels were determined using Freestyle Blood Glucose Test Strips and a handheld Freestyle Freestyle Blood Glucose Monitoring System (Abbott Laboratories,

USA). FBG measurements were normalized to those taken pre-treatment and percent change in FBG was plotted as a function of time post-treatment in GraphPad Prism 5. The AUC for change in FBG was integrated from 0-480 min and the corresponding value was plotted as a function of treatment.

Kinetic Assays and Inhibition Constant Determination. Kinetic analysis was performed to determine if modified peptides bind at the active-site of DPP IV. Purified recombinant human DPP IV (2 nM) was incubated with varying concentrations (10 to 80 μM) of native or P1'-modified peptides in DPP IV assay buffer for 30 min at RT in a 96-well plate. Control wells were incubated with vehicle (ddH_20) instead of peptide. Following the incubation, varying concentrations (up to 800 μM) of the chromogenic substrate *H*-Ala-Pro-*p*NA (Bachem, USA) were added to the appropriate wells and the plate was incubated for an additional 30 min at 37 °C. The DPP IV-mediated release of *p*NA from the chromogenic substrate *H*-Ala-Pro-*p*NA was monitored by spectrophotometry on a PerkinElmer Victor X3 plate reader at 410 nm. The concentration, c, of substrate cleaved was calculated using Beer-Lambert's Law ($A = \varepsilon bc$), where A is the absorbance of *p*NA (410 nm), ε is the molar extinction of *p*NA (8800 M^{-1} cm^{-1}) and b is the pathlength. The initial enzyme velocity (nmol/sec) measured over the first 5 min of the reaction were plotted as a function of substrate concentration. For K_i determination, the data was fit to a mixed-model inhibition equation using GraphPad Prism software.

Supporting Information

Supporting Information Available: Safety study measurements (food and water intake, bodyweight, organ weights, comprehensive blood count and chemistry), CD spectra, kinetic data, ^1H NMR spectra, $t_{½}$ values, IC_{50} values, EC_{50} values, published K_m values, calculated K_i values, and the synthesis and

characterization of Fmoc-L-β, β-dimethyl-Asp(O-t-Bu)-OH (**4**) and Fmoc-β, β-dimethyl-Gln-Gly-OH (**5**). This material is available free of charge via the Internet at http://pubs.acs.org.

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Abbreviations: αLP (alpha-lytic protease), aminopeptidase P 2 (APP2), β-galactosidase (β-gal), fasting blood glucose (FBG), brain natriuretic peptide (BNP), confidence interval (CI), dipeptidyl peptidase IV (DPP IV), dipeptidyl peptidase 8 (DPP8), enterostatin (ENT), fibroblast activation protein alpha (FAPα), glucagon (GCG), glucagon-like peptide-1 (7-36) amide (GLP-1), glucose-dependent insulinotropic peptide (GIP), neuropeptide Y (NPY), noroleucine (Nle), oxyntomodulin (OXM), *para*-nitroanilide (*p*NA), trifluoroethanol (TFE), Tufts University Core Facility (TUCF), type 2 diabetes (T2D)

References

1. McGregor, D. P., Discovering and improving novel peptide therapeutics. *Curr Opin Pharmacol* **2008,** 8, (5), 616-619.

2. Saladin, P. M.; Zhang, B. D.; Reichert, J. M., Current trends in the clinical development of peptide therapeutics. *IDrugs* **2009,** 12, (12), 779-784.

3. Deacon, C. F.; Johnsen, A. H.; Holst, J. J., Degradation of glucagon-like peptide-1 by human plasma in vitro yields an N-terminally truncated peptide that is a major endogenous metabolite in vivo. *J Clin Endocrinol Metab* **1995,** 80, (3), 952-957.

4. Deacon, C. F.; Nauck, M. A.; Meier, J.; Hucking, K.; Holst, J. J., Degradation of endogenous and exogenous gastric inhibitory polypeptide in healthy and in type 2 diabetic subjects as revealed using a new assay for the intact peptide. *J Clin Endocrinol Metab* **2000,** 85, (10), 3575-3581.

5. Mentlein, R., Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides. *Regul Pept* **1999,** 85, (1), 9-24.

6. Pospisilik, J. A.; Hinke, S. A.; Pederson, R. A.; Hoffmann, T.; Rosche, F.; Schlenzig, D.; Glund, K.; Heiser, U.; McIntosh, C. H.; Demuth, H., Metabolism of glucagon by dipeptidyl peptidase IV (CD26). *Regul Pept* **2001,** 96, (3), 133-141.

7. Schjoldager, B. T.; Baldissera, F. G.; Mortensen, P. E.; Holst, J. J.; Christiansen, J., Oxyntomodulin: a potential hormone from the distal gut. Pharmacokinetics and effects on gastric acid and insulin secretion in man. *Eur J Clin Invest* **1988,** 18, (5), 499-503.

8. Grouzmann, E.; Fathi, M.; Gillet, M.; de Torrente, A.; Cavadas, C.; Brunner, H.; Buclin, T., Disappearance rate of catecholamines, total metanephrines, and neuropeptide Y from the plasma of patients after resection of pheochromocytoma. *Clin Chem* **2001,** 47, (6), 1075-1082.

9. Clerico, A.; Iervasi, G.; Mariani, G., Pathophysiologic relevance of measuring the plasma levels of cardiac natriuretic peptide hormones in humans. *Horm Metab Res* **1999,** 31, (9), 487-498.

10. Bouras, M.; Huneau, J. F.; Luengo, C.; Erlanson-Albertsson, C.; Tome, D., Metabolism of enterostatin in rat intestine, brain membranes, and serum: differential involvement of proline-specific peptidases. *Peptides* **1995,** 16, (3), 399-405.

11. Hojberg, P. V.; Vilsboll, T.; Rabol, R.; Knop, F. K.; Bache, M.; Krarup, T.; Holst, J. J.; Madsbad, S., Four weeks of near-normalisation of blood glucose improves the insulin response to glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide in patients with type 2 diabetes. *Diabetologia* **2009,** 52, (2), 199-207.

12. Nauck, M. A.; Kleine, N.; Orskov, C.; Holst, J. J.; Willms, B.; Creutzfeldt, W., Normalization of fasting hyperglycaemia by exogenous glucagon-like peptide 1 (7-36 amide) in type 2 (non-insulin-dependent) diabetic patients. *Diabetologia* **1993,** 36, (8), 741-744.

13. Toft-Nielsen, M. B.; Madsbad, S.; Holst, J. J., Continuous subcutaneous infusion of glucagon-like peptide 1 lowers plasma glucose and reduces appetite in type 2 diabetic patients. *Diabetes Care* **1999,** 22, (7), 1137-1143.

14. Cohen, M. A.; Ellis, S. M.; Le Roux, C. W.; Batterham, R. L.; Park, A.; Patterson, M.; Frost, G. S.; Ghatei, M. A.; Bloom, S. R., Oxyntomodulin suppresses appetite and reduces food intake in humans. *J Clin Endocrinol Metab* **2003,** 88, (10), 4696-4701.

15. Day, J. W.; Ottaway, N.; Patterson, J. T.; Gelfanov, V.; Smiley, D.; Gidda, J.; Findeisen, H.; Bruemmer, D.; Drucker, D. J.; Chaudhary, N.; Holland, J.; Hembree, J.; Abplanalp, W.; Grant, E.; Ruehl, J.; Wilson, H.; Kirchner, H.; Lockie, S. H.; Hofmann, S.; Woods, S. C.;

Nogueiras, R.; Pfluger, P. T.; Perez-Tilve, D.; DiMarchi, R.; Tschop, M. H., A new glucagon and GLP-1 co-agonist eliminates obesity in rodents. *Nat Chem Biol* **2009,** 5, (10), 749-757.

16. Kerr, B. D.; Flatt, P. R.; Gault, V. A., (D-Ser2)Oxm[mPEG-PAL]: a novel chemically modified analogue of oxyntomodulin with antihyperglycaemic, insulinotropic and anorexigenic actions. *Biochem Pharmacol* 80, (11), 1727-1735.

17. Meier, J. J.; Gallwitz, B.; Askenas, M.; Vollmer, K.; Deacon, C. F.; Holst, J. J.; Schmidt, W. E.; Nauck, M. A., Secretion of incretin hormones and the insulinotropic effect of gastric inhibitory polypeptide in women with a history of gestational diabetes. *Diabetologia* **2005,** 48, (9), 1872-1881.

18. Meneilly, G. S.; Bryer-Ash, M.; Elahi, D., The effect of glyburide on beta-cell sensitivity to glucose-dependent insulinotropic polypeptide. *Diabetes Care* **1993,** 16, (1), 110-114.

19. Wynne, K.; Park, A. J.; Small, C. J.; Meeran, K.; Ghatei, M. A.; Frost, G. S.; Bloom, S. R., Oxyntomodulin increases energy expenditure in addition to decreasing energy intake in overweight and obese humans: a randomised controlled trial. *Int J Obes (Lond)* **2006,** 30, (12), 1729-1736.

20. Wynne, K.; Park, A. J.; Small, C. J.; Patterson, M.; Ellis, S. M.; Murphy, K. G.; Wren, A. M.; Frost, G. S.; Meeran, K.; Ghatei, M. A.; Bloom, S. R., Subcutaneous oxyntomodulin reduces body weight in overweight and obese subjects: a double-blind, randomized, controlled trial. *Diabetes* **2005,** 54, (8), 2390-2395.

21. Erlanson-Albertsson, C.; York, D., Enterostatin--a peptide regulating fat intake. *Obes Res* **1997,** 5, (4), 360-372.

22. Lin, L.; Park, M.; York, D. A., Enterostatin inhibition of dietary fat intake is modulated through the melanocortin system. *Peptides* **2007,** 28, (3), 643-649.

23. Mei, J.; Erlanson-Albertsson, C., Effect of enterostatin given intravenously and intracerebroventricularly on high-fat feeding in rats. *Regul Pept* **1992,** 41, (3), 209-218.

24. Redrobe, J. P.; Dumont, Y.; St-Pierre, J. A.; Quirion, R., Multiple receptors for neuropeptide Y in the hippocampus: putative roles in seizures and cognition. *Brain Res* **1999,** 848, (1-2), 153-166.

25. Thorsell, A.; Karlsson, R. M.; Heilig, M., NPY in alcoholism and psychiatric disorders. *Exs* **2006**, (95), 183-192.

26. Thorsell, A.; Slawecki, C. J.; El Khoury, A.; Mathe, A. A.; Ehlers, C. L., The effects of social isolation on neuropeptide Y levels, exploratory and anxiety-related behaviors in rats. *Pharmacol Biochem Behav* **2006,** 83, (1), 28-34.

27. Cheng, J. W., Nesiritide: review of clinical pharmacology and role in heart failure management. *Heart Dis* **2002,** 4, (3), 199-203.

28. Grantham, J. A.; Borgeson, D. D.; Burnett, J. C., Jr., BNP: pathophysiological and potential therapeutic roles in acute congestive heart failure. *Am J Physiol* **1997,** 272, (4 Pt 2), R1077-1083.

29. Sonnichsen, F. D.; Van Eyk, J. E.; Hodges, R. S.; Sykes, B. D., Effect of trifluoroethanol on protein secondary structure: an NMR and CD study using a synthetic actin peptide. *Biochemistry* **1992,** 31, (37), 8790-8798.

30. Bjelke, J. R.; Kanstrup, A. B.; Rasmussen, H. B., Selectivity among dipeptidyl peptidases of the S9b family. *Cell Mol Biol (Noisy-le-grand)* **2006,** 52, (4), 3-7.

31. Keane, F. M.; Nadvi, N. A.; Yao, T. W.; Gorrell, M. D., Neuropeptide Y, B-type natriuretic peptide, substance P and peptide YY are novel substrates of fibroblast activation protein-alpha. *Febs J* 278, (8), 1316-1332.

32. Mentlein, R.; Roos, T., Proteases involved in the metabolism of angiotensin II, bradykinin, calcitonin gene-related peptide (CGRP), and neuropeptide Y by vascular smooth muscle cells. *Peptides* **1996,** 17, (4), 709-720.

33. Brandt, I.; Lambeir, A. M.; Ketelslegers, J. M.; Vanderheyden, M.; Scharpe, S.; De Meester, I., Dipeptidyl-peptidase IV converts intact B-type natriuretic peptide into its des-SerPro form. *Clin Chem* **2006,** 52, (1), 82-87.

34. Mentlein, R.; Dahms, P.; Grandt, D.; Kruger, R., Proteolytic processing of neuropeptide Y and peptide YY by dipeptidyl peptidase IV. *Regul Pept* **1993,** 49, (2), 133-144.

35. Mentlein, R.; Gallwitz, B.; Schmidt, W. E., Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum. *Eur J Biochem* **1993,** 214, (3), 829-835.

36. Green, B. D.; Gault, V. A.; Irwin, N.; Mooney, M. H.; Bailey, C. J.; Harriott, P.; Greer, B.; Flatt, P. R.; O'Harte, F. P., Metabolic stability, receptor binding, cAMP generation, insulin secretion and antihyperglycaemic activity of novel N-terminal Glu9-substituted analogues of glucagon-like peptide-1. *Biol Chem* **2003,** 384, (12), 1543-1551.

37. Green, B. D.; Mooney, M. H.; Gault, V. A.; Irwin, N.; Bailey, C. J.; Harriott, P.; Greer, B.; Flatt, P. R.; O'Harte, F. P., Lys9 for Glu9 substitution in glucagon-like peptide-1(7-36)amide confers dipeptidylpeptidase IV resistance with cellular and metabolic actions similar to those of established antagonists glucagon-like peptide-1(9-36)amide and exendin (9-39). *Metabolism* **2004,** 53, (2), 252-259.

38. Gault, V. A.; Hunter, K.; Irwin, N.; Green, B. D.; Greer, B.; Harriott, P.; O'Harte, F. P.; Flatt, P. R., Characterisation and biological activity of Glu3 amino acid substituted GIP receptor antagonists. *Arch Biochem Biophys* **2007,** 461, (2), 263-274.

39. Druce, M. R.; Minnion, J. S.; Field, B. C.; Patel, S. R.; Shillito, J. C.; Tilby, M.; Beale, K. E.; Murphy, K. G.; Ghatei, M. A.; Bloom, S. R., Investigation of structure-activity relationships of Oxyntomodulin (Oxm) using Oxm analogs. *Endocrinology* **2009,** 150, (4), 1712-1722.

40. Santoprete, A.; Capito, E.; Carrington, P. E.; Pocai, A.; Finotto, M.; Langella, A.; Ingallinella, P.; Zytko, K.; Bufali, S.; Cianetti, S.; Veneziano, M.; Bonelli, F.; Zhu, L.; Monteagudo, E.; Marsh, D. J.; Sinharoy, R.; Bianchi, E.; Pessi, A., DPP-IV-resistant, long-acting oxyntomodulin derivatives. *J Pept Sci* 17, (4), 270-280.

41. Al-Sabah, S.; Donnelly, D., The positive charge at Lys-288 of the glucagon-like peptide-1 (GLP-1) receptor is important for binding the N-terminus of peptide agonists. *FEBS Lett* **2003,** 553, (3), 342-346.

42. Al-Sabah, S.; Donnelly, D., A model for receptor-peptide binding at the glucagon-like peptide-1 (GLP-1) receptor through the analysis of truncated ligands and receptors. *Br J Pharmacol* **2003,** 140, (2), 339-346.

43. Cabrele, C.; Beck-Sickinger, A. G., Molecular characterization of the ligand-receptor interaction of the neuropeptide Y family. *J Pept Sci* **2000,** 6, (3), 97-122.

44. Mimeault, M.; De Lean, A.; Lafleur, M.; Bonenfant, D.; Fournier, A., Evaluation of conformational and binding characteristics of various natriuretic peptides and related analogs. *Biochemistry* **1995,** 34, (3), 955-964.

45. Xiao, Q.; Giguere, J.; Parisien, M.; Jeng, W.; St-Pierre, S. A.; Brubaker, P. L.; Wheeler, M. B., Biological activities of glucagon-like peptide-1 analogues in vitro and in vivo. *Biochemistry* **2001,** 40, (9), 2860-2869.

46. Hinke, S. A.; Manhart, S.; Speck, M.; Pederson, R. A.; Demuth, H. U.; McIntosh, C. H., In depth analysis of the N-terminal bioactive domain of gastric inhibitory polypeptide. *Life Sci* **2004,** 75, (15), 1857-1870.

47. Gault, V. A.; O'Harte, F. P.; Harriott, P.; Flatt, P. R., Characterization of the cellular and metabolic effects of a novel enzyme-resistant antagonist of glucose-dependent insulinotropic polypeptide. *Biochem Biophys Res Commun* **2002,** 290, (5), 1420-1426.

48. Baldissera, F. G.; Holst, J. J.; Knuhtsen, S.; Hilsted, L.; Nielsen, O. V., Oxyntomodulin (glicentin-(33-69)): pharmacokinetics, binding to liver cell membranes, effects on isolated perfused pig pancreas, and secretion from isolated perfused lower small intestine of pigs. *Regul Pept* **1988,** 21, (1-2), 151-166.

49. Gros, L.; Thorens, B.; Bataille, D.; Kervran, A., Glucagon-like peptide-1-(7-36) amide, oxyntomodulin, and glucagon interact with a common receptor in a somatostatin-secreting cell line. *Endocrinology* **1993,** 133, (2), 631-638.

50. Runge, S.; Gram, C.; Brauner-Osborne, H.; Madsen, K.; Knudsen, L. B.; Wulff, B. S., Three distinct epitopes on the extracellular face of the glucagon receptor determine specificity for the glucagon amino terminus. *J Biol Chem* **2003,** 278, (30), 28005-28010.

51. Beck-Sickinger, A. G.; Wieland, H. A.; Wittneben, H.; Willim, K. D.; Rudolf, K.; Jung, G., Complete L-alanine scan of neuropeptide Y reveals ligands binding to Y1 and Y2 receptors with distinguished conformations. *Eur J Biochem* **1994,** 225, (3), 947-958.

52. Grandt, D.; Schimiczek, M.; Rascher, W.; Feth, F.; Shively, J.; Lee, T. D.; Davis, M. T.; Reeve, J. R., Jr.; Michel, M. C., Neuropeptide Y 3-36 is an endogenous ligand selective for Y2 receptors. *Regul Pept* **1996,** 67, (1), 33-37.

53. Kuhn-Wache, K.; Bar, J. W.; Hoffmann, T.; Wolf, R.; Rahfeld, J. U.; Demuth, H. U., Selective inhibition of dipeptidyl peptidase 4 by targeting a substrate-specific secondary binding site. *Biol Chem* 392, (3), 223-231.

54. Yoshimoto, T.; Orawski, A. T.; Simmons, W. H., Substrate specificity of aminopeptidase P from Escherichia coli: comparison with membrane-bound forms from rat and bovine lung. *Arch Biochem Biophys* **1994,** 311, (1), 28-34.

55. Aertgeerts, K.; Levin, I.; Shi, L.; Snell, G. P.; Jennings, A.; Prasad, G. S.; Zhang, Y.; Kraus, M. L.; Salakian, S.; Sridhar, V.; Wijnands, R.; Tennant, M. G., Structural and kinetic analysis of the substrate specificity of human fibroblast activation protein alpha. *J Biol Chem* **2005,** 280, (20), 19441-19444.

56. Everill, P.; Sudmeier, J. L.; Bachovchin, W. W., Direct NMR observation and pKa determination of the Asp102 side chain in a serine protease. *J Am Chem Soc* 134, (4), 2348-2354.

57. Tibaduiza, E. C.; Chen, C.; Beinborn, M., A small molecule ligand of the glucagon-like peptide 1 receptor targets its amino-terminal hormone binding domain. *J Biol Chem* **2001,** 276, (41), 37787-37793.

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