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Research Article

Preptin Analogues: Chemical Synthesis, Secondary Structure and Biological Studies

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Peptide hormones that modulate insulin secretion have been recognized to have therapeutic potential, with peptides such as amylin (pramlintide acetate, Symlin) and exendin-4 (exenatide, Byetta) now commercially available. Preptin is a peptide that has been shown to increase insulin secretion *in vitro* and *in vivo*. Here, we describe the first chemical synthesis and analysis of a short series of preptin analogues based on the rat preptin sequence. Phe 21 in the preptin sequence was substituted with the non-protein amino acids D-Phe, D-Hphe, 3-aminobenzoic acid and 1-aminocyclooctane-1-carboxylic acid, which rendered the preptin analogues resistant to chymotryptic protease hydrolysis at this position. Substitution of Phe 21 with these non-protein amino acids did not abrogate the insulin secretory effect of preptin, with analogues showing a similar dose-dependent effect on insulin secretion from βTC6-F7 mouse β-cells in both the presence and absence of glucose as unmodified rat preptin. Further studies on the stability of the preptin analogues and their effect on insulin secretion are in progress.

Key words: circular dichroism, insulin secretion, non-protein amino acids, preptin analogues, solid-phase peptide synthesis

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Preptin is a 34-residue polypeptide first isolated from the mouse pancreatic β-cell line βTC6-F7 (1). This peptide is derived from the E-domain of pro-IGF-II, a precursor to mitogenic insulin-like growth factor II (IGF-II), and the sequence is highly conserved between human and rodent species (Figure 1A). Preptin was found to enhance insulin secretion following glucose stimulation in cultured β-cells, in the isolated perfused rat pancreas and *in vivo* in the rat (1,2). Studies have positively correlated elevated preptin levels in patients with metabolic disturbances including gestational diabetes mellitus, polycystic ovary syndrome, type 2 diabetes mellitus and impaired glucose tolerance (3–5). In addition, the peptide was discovered to be osteogenic (6,7), reducing osteoblast apoptosis via actions involving the MAP-kinase pathway (6), and lower circulating preptin levels have been correlated with low bone mineral densities in male patients with osteoporosis and osteopaenia (8). Preptin is cleaved by endogenous proteases at phenylalanine 21 and has a half-life of 5 min *in vivo* (9). Cleavage at this point likely impacts the biological activity of preptin as a truncated preptin peptide (preptin 1–16) has been shown to have no effect on insulin secretion (10). Despite its therapeutic potential, there are no literature reports on the development of preptin analogues as potential drug candidates. In this article, we describe our studies on the first chemical synthesis and analysis of a short series of preptin analogues based on the rat preptin sequence (Figure 1A). Phe 21 in the preptin sequence was substituted by the non-protein amino acids D-Phe, D-Hphe, 3-aminobenzoic acid and 1-aminocyclooctane-1-carboxylic acid (Figure 1B) in an attempt to overcome the proteolytic susceptibility of preptin at this position, and biological activity was measured by determining the effect of the peptides on insulin secretion from βTC6 F7, a mouse β-cell line. Further studies on the stability of the preptin analogues and their effect on insulin secretion are in progress.

Materials and Methods

Peptide synthesis and purification

The preptin analogues were synthesized manually on the solid phase following a standard Fmoc protocol. All peptides were assembled on preloaded N-Fmoc-Leu-Wang resin on polystyrene solid support having a substitution level of 0.3–0.8 mmol/g at 0.1 mmol scale. Actual substitution was determined spectrophotometrically as described below. The Fmoc amino acid resin (5–10 mg) was weighed into a tube to which 3 mL of 20% piperidine in DMF was added and gently agitated for 20 min. The resin was filtered off and the absorbance of the filtrate measured at 290 nm against the blank. Loading was

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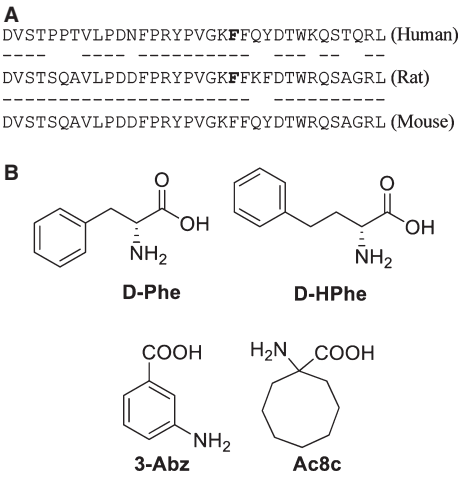

Figure 1: (A) Amino acid sequences of human and rat preptins compared with the mouse sequence. The target phenylalanine at residue 21 is shown in bold, and dashed lines indicate the areas of homology. (B) The chemical structures of the non-protein amino acids used in the design of the preptin analogues.

calculated using the equation – loading (mmol/g) = (absorbance$_{sample}$)/(mg of sample × 1.75). In a typical coupling procedure, four equivalents of the Fmoc amino acid were dissolved in 0.5 M coupling reagent solution (3.8 eq.) in DMF followed by addition of DIPEA (10 eq.). The reaction mixture was agitated for 1 h at room temperature after which the liquid was drained off, the resin thoroughly washed with DMF (5 × 5 mL) and treated with 5 mL of 20% v/v of piperidine in DMF twice (1 × 5 min and 1 × 20 min) with gentle agitation. The piperidine solution was drained off and the resin thoroughly washed with DMF (5 × 5 mL). Peptide chain elongation was continued following the above protocol of amino acid coupling and Fmoc deprotection until the entire sequence was assembled. The side-chain protecting groups used in the syntheses were *tert*-butyl (*t*Bu) for serine and threonine, *tert*-butoxycarbonyl (Boc) for lysines and tryptophan, *tert*-butoxy (*t*BuO) for aspartic and glutamic acids, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for arginine and trityl (Trt) for histidine, glutamine and asparagines.

The coupling reactions were monitored using the Kaiser test (11). Unsuccessful couplings, indicated by the presence of purple beads in the Kaiser test, were repeated without deprotection of the Fmoc group on the amino acid until the Kaiser test gave negative results. Once the desired peptide chains were assembled, the N-terminal Fmoc protecting group was removed by washing the resin with 20% piperidine in DMF for two consecutive times of 5 and 20 min, respectively. The peptidyl resin was then thoroughly washed with DMF and DCM and dried overnight in the desiccator. The final peptides were cleaved from the resin using 10 mL of the TFA cocktail mixture: (TFA:TIS:H_2O 95:2.5:2.5; v/v/v) for 3 h with gentle agitation. The cleavage mixture was evaporated under nitrogen, and the peptides were precipitated using excess cold diethyl ether. The crude, white products were resuspended in cold diethyl ether, centrifuged thrice decanting ether each time. The crude products were redissolved in 80:20 (v/v) water/acetonitrile and lyophilized to obtain the peptides as white, fluffy solids.

The crude peptides were purified using reversed-phase HPLC on GE Pharmacia ÄKTApurifier 10 system on a Phenomenex Luna C_{18} 100 Å (250 × 10 mm; particle size 5 micron) column using gradient elution with 0.1% TFA in water/acetonitrile at a flow rate of 4 or 5 mL/min (as indicated on the respective traces). A linear gradient of 10–60% solvent B over 30 min was used at the indicated flow rates, where A and B were 0.1% TFA-H_2O and 80% CH_3CN/0.1% TFA-H_2O (80:20 v/v), respectively. Peptides were detected at 214 nm (amide) and 280 nm (aromatic). The purity and identity of the peptides were established by analytical HPLC and ESIMS mass spectrometry. Analytical HPLC was performed on Phenomenex Jupiter C_{18} column 300 Å (250 × 2.0 mm, particle size 5 micron) using acetonitrile and water containing 0.1% TFA as the mobile phase. A linear gradient of 10–80% B over 35 min at a flow rate of 0.3 mL/min was used where A and B were 0.1% TFA-H_2O and 80:20 v/v CH_3CN/0.08% TFA-H_2O.

Fmoc protection of non-protein amino acids

(R)-2-(9-fluorenylmethyloxycarbonylamino)-4-phenylbutanoicacid

(*R*)-2-amino-4-phenylbutyric acid (1.0 g, 5.58 mmol) was dissolved in the minimum volume of 1,4-dioxane:10% Na_2CO_3/water (1:2) mixture. To the mixture, Fmoc-OSu (2.82 g, 8.37 mmol) dissolved in 1,4-dioxane was added dropwise. Stirring was maintained over 24 h at room temperature to form a milky white solution. The precipitate was dissolved in water and washed with diethyl ether thrice. The aqueous layer was acidified to pH 3 at 0 °C to form a white precipitate, which was extracted using ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford (*R*)-2-(9-fluorenylmethyloxycarbonylamino)-4-phenylbutanoic acid as a brown solid (1.606 g, 71.7%). HRMS–ESI (*m/z*): $[M + H]^+$ calculated for $C_{25}H_{24}NO_4$, 402.1700; observed, 402.1710; $[M + Na]^+$ calculated for $C_{25}H_{23}NO_4Na$, 424.1519; observed, 424.1535.

3-(9-fluorenylmethyloxycarbonyl)aminobenzoic acid

3-aminobenzoic acid (0.5 g, 3.65 mmol) was dissolved in the minimum volume of water/acetonitrile (1:1) mixture. Fmoc-OSu (1.85 g, 5.47 mmol) was added followed by DIPEA (0.645 mL) at 60 °C. The reaction mixture was stirred for 24 h to form a white precipitate. The mixture was diluted with water, and the aqueous layer was extracted with ethyl acetate. The organic layer was evaporated

under reduced pressure to afford a yellow solid (0.9483 g, 72.3%). HRMS–ESI (*m/z*): $[M + H]^+$ calculated for $C_{22}H_{18}NO_4$, 360.12; observed, 360.12; $[M + Na]^+$ calculated for $C_{22}H_{17}NO_4Na$, 382.1050; observed, 382.1039.

CD spectroscopy

Circular dichroism (CD) spectra were recorded on a Pi-Star-180 (Applied Photophysics, Surrey, UK) spectrometer at ambient temperature in 1 mm cuvettes in the interval from 180 to 250 nm in phosphate buffer (5 mM) as well as in 50% TFE/H_2O mixtures at pH 7 at a peptide concentration of 50 μM. The scanning speed for each spectrum was 300 points per minute. Peptide stock solutions were prepared by weight in buffer or 50% TFE/water, as desired, assuming water content of 25% for the lyophilized peptides, and diluted to 50 μM concentration using the respective solvents for CD studies.

Chymotryptic stability assay

A volume of 20 μL of preptin and analogues (1 mg/mL in MilliQ water) was mixed with 20 μL of chymotrypsin (25 μg/mL in 50 mM ammonium bicarbonate; Roche Sequencing Grade) and incubated at 37 °C. Aliquots of 1 μL were taken at 1, 2, 5, 10, 30, 60 and 120 min and loaded directly onto a stainless steel MALDI plate, preseeded with CHCA matrix (10 mg/mL in 50:50 MeCN:0.1% TFA). The samples were analysed by MALDI-TOF MS (Voyager DE Pro; Applied Biosystems).

Preptin-stimulated insulin secretion

βTC6-F7 cells were kind gift from Prof Garth Cooper (originally obtained from Dr Shimon Efrat). Cells were subcultured (passage 52–60; 24-well plates; 4×10^5 cells/well), grown to 80% confluence and washed twice in Krebs Ringer buffer (KRB)/HEPES, pH 7.4. Preptin was serially diluted in incubation medium (KRB/HEPES containing 0.1% BSA) ±10 mM glucose to final concentrations of 50, 150, 600, 1200, 2400 nM, before 500-μL volumes were added to quadruplicate wells. Cells were incubated (at 37 °C for 2 h), medium aspirated and centrifuged (16 000 g for 3 min), and the supernatant was analysed for insulin (AlphaLISA; Perkin Elmer, Santa Clara, CA, USA). Statistical analysis was undertaken using one-way ANOVA with post hoc analysis using Dunnett's multiple comparison test.

Results and Discussion

Analogue design and peptide synthesis

The sequences of human and rat preptin and the chemical structures of the non-protein amino acids used to substitute for Phe 21 in preptin analogues are shown in Figure 1. Phenylalanine is a hydrophobic as well as aromatic amino acid. Therefore, in the analogues, Phe 21 was substituted by non-protein amino acids that retain either or both of these properties. We replaced Phe 21 with its non-natural isomer D-Phe, its higher homologue, D-Hphe, 3-amino benzoic acid, as well as 1-aminocyclooctane-1-carboxylic acid (Ac_8c), which belongs to the class of α,α-dialkyl glycines. The D-amino acid analogues of preptin are expected to have longer half-lives because peptides made up of D-amino acids are particularly resistant to enzymatic degradation (12–14). D-amino acids have also been reported to increase receptor/acceptor interactions *in vivo* by stabilizing the secondary structure in many peptides (15). The D-isomer of Hphe is expected to enhance the stability of the peptide towards enzymatic degradation while maintaining the aromatic nature of Phe itself. The aminobenzoic acid was chosen because of its structural similarity to L-Phe. Ac_8c, which belongs to the class of α,α-dialkyl glycines, was included, because of our ongoing interest in this group of amino acids, which have the ability to promote specific secondary structures in designed peptides (16,17). Even though Ac_8c does not completely mimic Phe, being hydrophobic and having the same number of carbon atoms as in Phe (nine carbons in total), the Ac_8c analogue would have similar hydrophobicity to the unmodified sequence. It was also expected that the Ac_8c peptide would adopt a stable secondary structure, which may help to enhance the biological activity of the peptide. Synthetic details of the preptin analogues are summarized in Table 1. The syntheses of all peptides were successful under all coupling conditions, even though significant differences were observed in the purity of the final product depending on the coupling reagent used (refer Figures 2 and 3 and Table 1).

Attempted syntheses using HBTU as the coupling reagent

Initial syntheses of rat and human preptins were attempted using *N*-[(1H-benzotriazol-l-yl)(dimethylamino) methylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HBTU) as coupling reagent. While most of the Fmoc-protected amino acids underwent successful couplings, a few amino acids such as arginine-15 failed to couple in the first attempt as indicated by a positive Kaiser test. Such couplings were repeated until the Kaiser test was negative.

Table 1: Summary of the synthesis of wild-type preptin and its analogues

Species	Analogue	Coupling reagent	% yield	Crude % purity
Human	Unmodified	HBTU	80	20
Rat	Unmodified	HBTU	65	27
Rat	Unmodified	HATU	95	40
Rat	D-Phe	HCTU	88	40
Rat	D-Hphe	HCTU	83	53
Rat	3-abz-OH	HATU	95	41
Rat	Ac_8c	HCTU	90	12

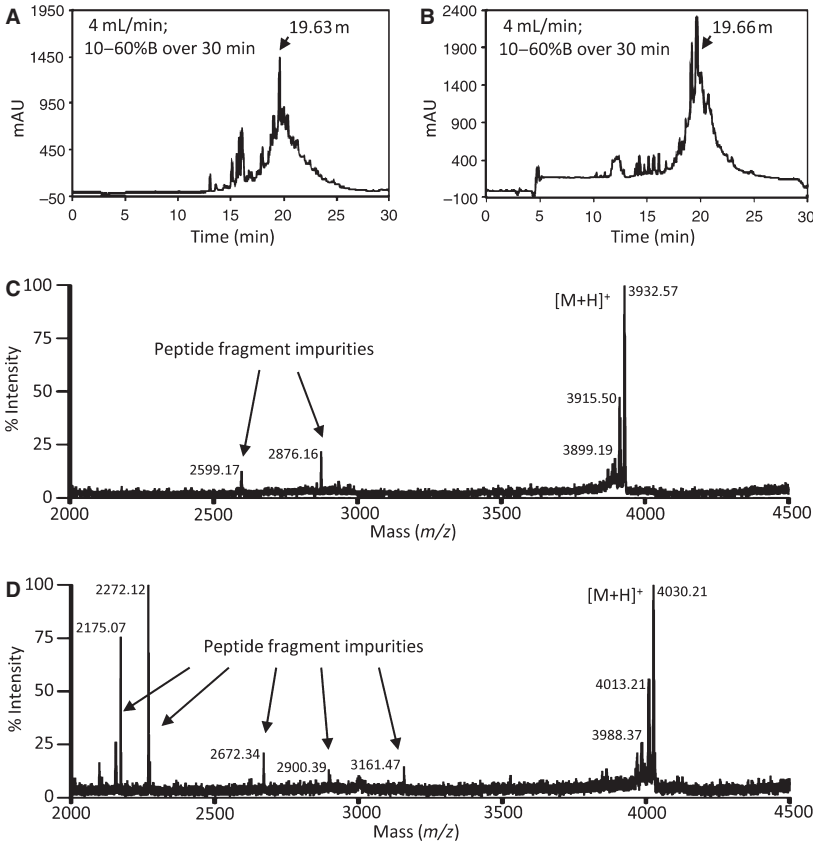

Figure 2: Semi-preparative HPLC traces of HBTU synthesis of (A) rat preptin (B) human preptin, and MALDI-TOF spectra of the main peaks from (C) rat and (D) human preptins. The main rat and human preptin peak signals are shown as $[M + H]^+$, and peptide fragment impurities are indicated by arrows.

Crude yields of rat and human preptin were 65 and 80%, respectively. The HPLC profiles of crude rat and human preptin showed several overlapping peaks (Figure 2A,B). The MS analysis of the peak at 19.63 min of rat preptin (Figure 2C) revealed the presence of impurities arising from incomplete couplings and deprotections during the synthesis. Attempts to repurify the peak to homogeneity were unsuccessful. MALDI-MS analysis of the major peak eluting at 19.66 min of crude human preptin (Figure 2D) showed signals at m/z 2272.1187 and 2175.0738 corresponding to peptide termination at Pro 18 and Val 17, respectively, and several other deletion peptides. Similar to rat preptin, the desired level of purity for the human preptin was unachievable using HBTU as the coupling reagent.

Improved syntheses

Coupling reagents play an important role in the efficiency of solid-phase peptide synthesis. HBTU, used in the previous syntheses, is the reagent of choice for SPPS (18,19) and is a better reagent to N,N'-dicyclohexylcarbodiimide (DCC) and N,N'-diisopropylcarbodiimide (DIC) which are known to cause epimerization during synthesis (20,21). However, coupling times with HBTU are long (1 h), which is undesirable in the manual synthesis of peptides. Since its development, HBTU has been reported to have low coupling yields, especially when coupling sterically hindered amino acids and synthesizing 'difficult sequences' (22). Low purity and poor yields from the previous syntheses of preptin may be attributed to these drawbacks of HBTU. Therefore, we trialled two, reportedly more efficient, coupling reagents – 2-(1H-azabenzotriazole-1-yl)-1, 1, 3, 3-tetramethyl-uronium hexafluorophosphate (HATU) and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate) (HCTU) for the syntheses of the remaining preptin analogues. HATU has been shown to be superior to HBTU (23) particularly for difficult peptides. The coupling reaction times are reduced to 30 min per amino acid with HATU, thus minimizing the likelihood of racemization. Both rat and human preptin were resynthesized using HATU as the coupling reagent. The resynthesized rat (R_t: 17.99 min, Figure 3A) and human preptin (R_t: 21.84 min, Figure 3B) were afforded at 95% overall yield. Both peptides were purified to homogeneity efficiently. Representative analytical HPLC chromatograms of purified human and rat preptin are shown (Figure 3C,D).

For analogue syntheses, it was decided to focus on rat preptin, as our initial bioactivity assays indicated that human preptin and analogues were not able to increase insulin secretion from the mouse βTC6 F7 cell line, we were using as our target model. HATU was used for the 3-abz analogue synthesis, while the other three preptin peptides were synthesized using HCTU as the coupling reagent (refer Table 1). HCTU is known to have a comparable coupling efficiency to HATU, but is available at a more affordable price. HCTU is a 6-Cl-HOBt derivative

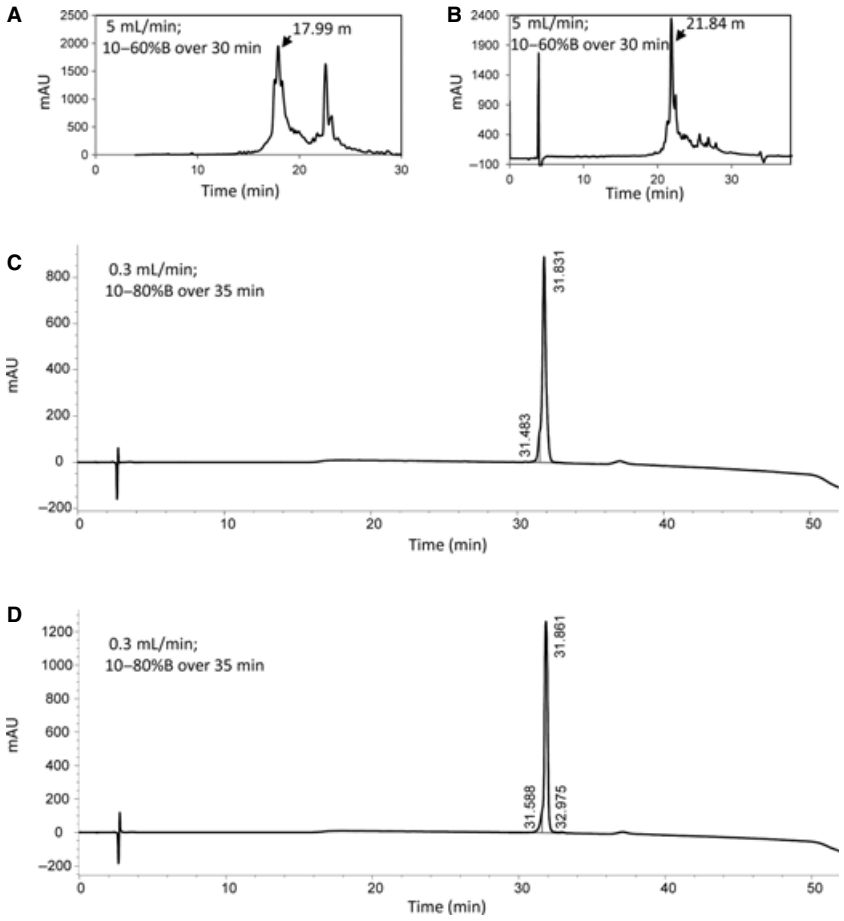

Figure 3: Semi-preparative HPLC traces of improved synthesis of (A) rat and (B) human preptins using HATU as the coupling reagent. Representative analytical chromatograms of the main peaks from (C) rat and (D) human preptins.

and its chemical reactivity is credited to the chloro-substituted aromatic ring (24,25). The 3-abz analogue was afforded at 90% yield while the D-HPhe and D-Phe analogues resulted in crude yields of 83% and 88%, respectively. The Ac_8c analogue exhibited some deletion peptides. This was not surprising given the steric hindrance imposed by the bulky cyclooctane ring. Reaction times for both Fmoc-Ac_8c and the next N-terminal residue, Fmoc-Lys were extended to 1.5 and 1 h, respectively, to ensure complete acylation. Clearly, steric hindrance imposed by the Ac_8c hampered the acylation of subsequent amino acids towards the N-terminus. The crude product was afforded at 90% yield, and while the semi-preparative HPLC chromatogram showed many other peptide impurities arising from sequence terminations alongside the peptide of interest, HPLC purification afforded the peptide in 96.1% purity for further biological and structural analysis (refer Table 2); hence, HATU or other coupling reagents were not further trialled for the synthesis of this peptide.

Circular dichroism spectroscopy

The secondary structure of preptin peptides was investigated using circular dichroism (CD) spectroscopy. CD spectra of human and rat preptins and rat analogues were recorded between 180 and 250 nm in phosphate buffer (5 mM) and in 50:50 TFE/H_2O, and the units were converted to molar ellipticity [θ]. The spectra are shown in Figure 4, and the $[\theta]_{222}/[\theta]_{208}$ of peptides in TFE/H_2O are provided in Table 3.

The negative minima at around 200 nm were a common characteristic of all the peptides in phosphate buffer, indicating random coil conformation. This may be attributed to the multiple prolines in these sequences, which are known to be helix breakers (26).

In samples with 50% TFE, there is a mixture of both helix and random coil conformations in all the peptides. The characteristic features of an α-helical CD spectrum are two negative minima at 222 and 208 nm (27). A ratio between $[\theta]_{222}/[\theta]_{208}$ is a measure of the helicity a peptide exhibits in solution. For an ideal α-helix, the $[\theta]_{222}/[\theta]_{208}$ ratio has been predicted to be 1. For a 3_{10}-helix, the ratio $[\theta]_{222}/[\theta]_{208}$ is $\sim$0.5 (28,29).

The $[\theta]_{222}/[\theta]_{208}$ ratios listed in Table 3 indicate that none of the peptides form an ideal helix even in the presence of 50% TFE. Among all peptides studied, the Ac_8C rat preptin has the highest degree of helicity in the presence of 50% TFE, reiterating the propensity of Ac_8c to promote helical conformation in designed peptides.

Table 2: Physical characteristics of the preptin peptides

Species	Analogue	HPLC t_R (min) and % purity	Formula	M_{calcd} (Da)	$M_{observed}$ (Da)
Human	Unmodified	31.86; 93.0%	$C_{187}H_{275}N_{47}O_{53}$	4029.03	4029.07
Rat	Unmodified	31.83; 95.2%	$C_{181}H_{268}N_{48}O_{51}$	3931.99	3932.01
Rat	D-Phe	31.99; 94.7%	$C_{181}H_{268}N_{48}O_{51}$	3931.99	3932.02
Rat	D-Hphe	32.85; 94.1%	$C_{182}H_{270}N_{48}O_{51}$	3945.00	3946.03
Rat	3-abz-OH	30.94; 94.2%	$C_{179}H_{264}N_{48}O_{51}$	3903.96	3903.99
Rat	Ac_8c	33.50; 96.1%	$C_{181}H_{274}N_{48}O_{51}$	3938.04	3938.06

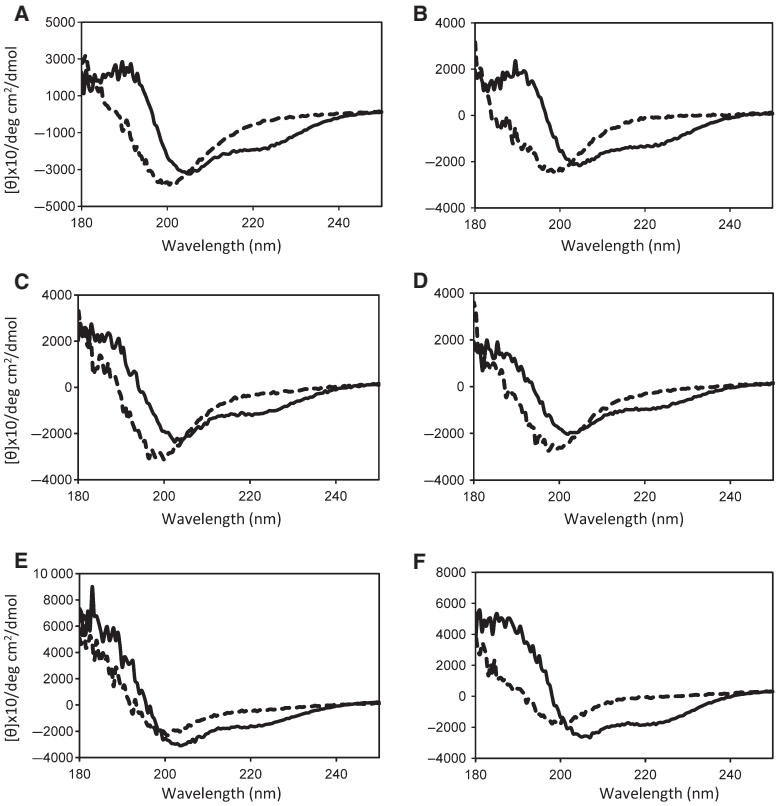

Figure 4: CD spectra of unmodified (A) human and (B) rat preptin, and rat preptin analogues: (C) D-Phe (D) D-Hphe (E) 3-abz-OH (F) Ac_8c at pH 7. The dotted lines are spectra in buffer, and solid lines are those in 50:50 TFE/buffer.

Table 3: The $[\theta]_{222}/[\theta]_{208}$ of preptin peptides in TFE:H_2O

Species	Analogue	$[\theta]$ 222 nm	$[\theta]$ 208 nm	$[\theta]_{222}/[\theta]_{208}$
Human	Unmodified	−18856.64	−28587.40	0.660
Rat	Unmodified	−13313.78	−19087.54	0.698
Rat	D-Phe	−11523.68	−17018.54	0.677
Rat	D-Hphe	−9134.70	−15566.22	0.587
Rat	3-abz-OH	−15384.06	−23925.20	0.643
Rat	Ac8c	−17395.06	−23754.00	0.732

Substituting non-protein amino acids at Phe 21 protects preptin analogues from proteolytic cleavage at this position

We chose to challenge our preptin molecules with chymotrypsin, as this protease is known to cleave at the C-terminus of aromatic amino acids including Phe (30). We incubated the unmodified and substituted preptin analogues with chymotrypsin at 37 °C for various time-points and monitored the composite molecular masses of the mixture by MALDI-TOF MS. For human and rat preptin, molecular masses equivalent to Asp 1-Phe 22 and Asp 1-Tyr 27 were observed after only 1-min incubation, while Asp 1-Phe 21 was observed after 2 and 10 min of incubation from rat and human preptin, respectively. In the rat preptin analogues, while molecular masses equivalent to Asp 1-Phe 22 and Asp 1-Tyr 27 were observed, no mass equivalent to Asp 1-Phe 21 was measured. Furthermore, the intact molecular mass for unmodified preptin had disappeared completely by 2 min of incubation, while residual amounts of the intact molecule were still apparent in the analogues after 5 min incubation (although these had disappeared by 10 min). Of further interest, we noted that rat D-Hphe did not produce Asp 1-Phe 22 until 30 min of incubation, whereas this species was present even after 1 min in the unmodified rat preptin. This suggests that substitu-

tion at Phe 21 with D-Hphe protects not only that position from chymotryptic digestion, but also the neighbouring Phe 22. Regardless, in all cases after 2 h of coincubation with chymotrypsin, the major product observed was Asp 1-Phe 22, while Asp 1-Phe 21 was never observed, confirming that modification of Phe 21 with the chosen non-protein amino acids did prevent hydrolysis at this position.

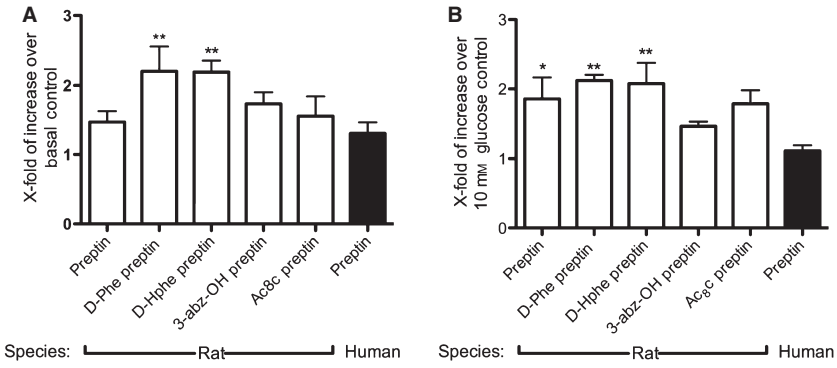

Figure 5: Stimulation of insulin secretion by rat preptin and analogues. Rat preptin and the 4 analogues (1200 nM) were compared alongside human preptin (black column) for their ability to augment insulin secretion from βTC6-F7 cells (A) in the absence and (B) presence of glucose, $n \geq 3$ for each condition. Asterisks indicate significance compared with basal or glucose-stimulated secretion ***p < 0.005, **p < 0.01, *p < 0.05.

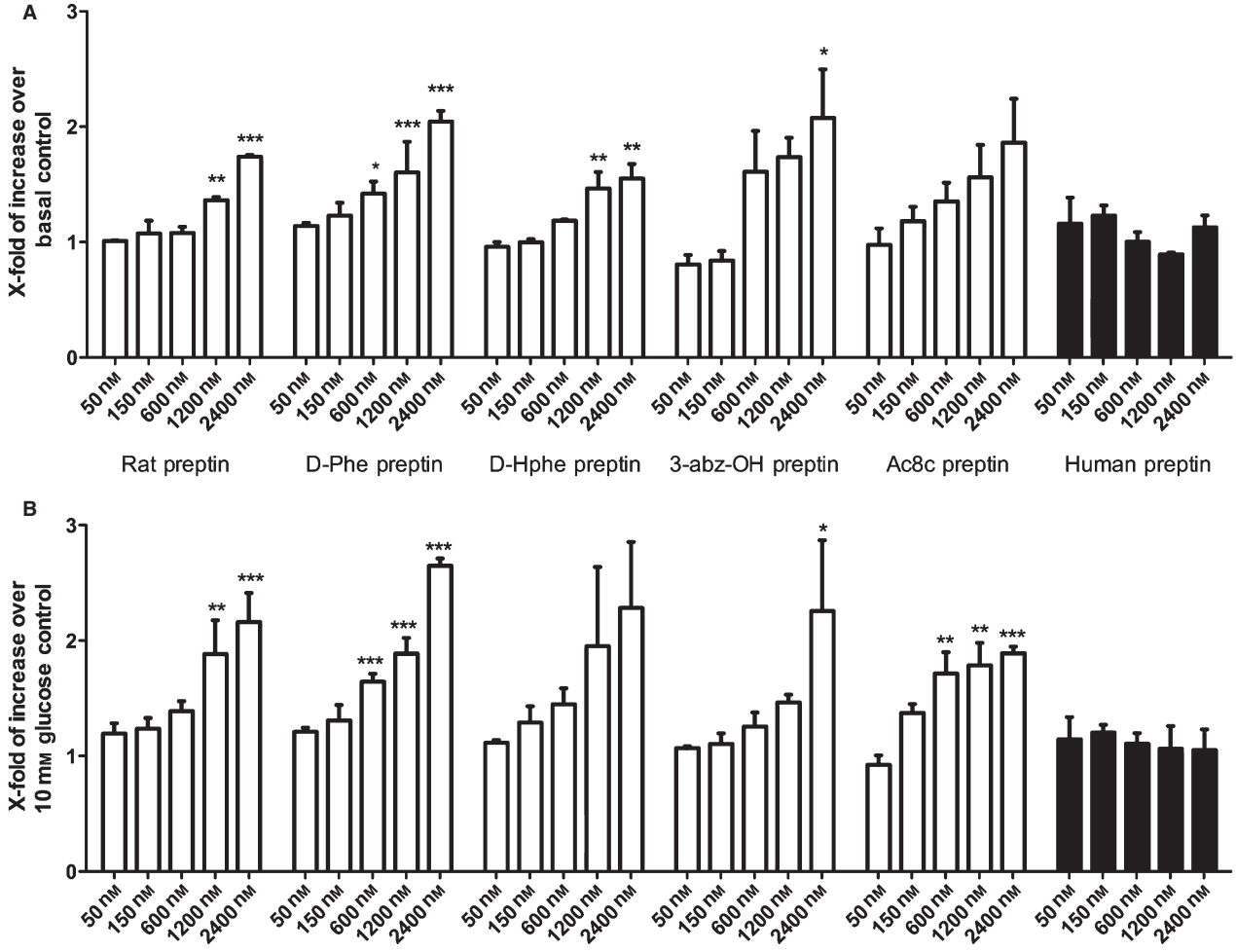

Figure 6: Dose–response curves showing the effects of preptin and analogues on insulin secretion from βTC6-F7 cells at nominated concentrations (A) in the absence and (B) presence of glucose, $n \geq 3$ for each condition. Asterisks indicate significance compared with basal or glucose-stimulated secretion ***p < 0.005, **p < 0.01, *p < 0.05.

Rat preptin analogues are effective at stimulating insulin secretion

Our earlier findings showed that rat preptin augmented insulin secretion from the isolated perfused rat pancreas and cultured mouse β-cells (βTC6-F7 cell line) in the presence of glucose (1), so we used the validated mouse βTC6-F7 cell insulin secretion assay to compare the bioactivity of preptin and substituted preptin analogues. Initially, we screened all the molecules in the presence and absence of glucose (Figure 5A,B). Here, we show for the first time that human preptin is not bioactive in this cell model, indicating that even though well conserved (see Figure 1A), the rodent molecules are more closely related and obviously the human peptide is sufficiently different to prevent cross-reaction with receptors in the mouse β-cell. We continued to incorporate human preptin in our assays because it acted as a good control, proving that the effects on insulin secretion are specifically due to the sequence of rat preptin and not due to some copurified artefact. It was interesting to note that two of the substituted analogues D-Phe and D-Hphe preptin had a significant effect on insulin secretion in both the presence and absence of glucose (Figure 5A,B). To confirm these observations, we undertook dose–response assays. Again rat preptin and the substituted analogues all showed a dose-dependent response in the presence and absence of glucose (Figure 6A,B), eliciting a twofold to 2.5-fold increase in insulin secretion above basal conditions in the absence and presence of 10 mM glucose, respectively. Once again, the rat D-Phe preptin appeared to stimulate the highest levels of insulin secretion; however, this was not significantly different to the unmodified preptin-stimulated response at the same concentrations.

Conclusion

A short series of preptin analogues were synthesized and analysed for the first time. The use of HBTU as the coupling reagent gave unsatisfactory results with the syntheses of the preptin series. The use of superior peptide coupling reagents such as HATU or HCTU gave improved synthesis of the peptide analogues. Our results also indicate that a cheaper reagent such as HCTU provides a comparable result to HATU in the manual solid-phase synthesis of long peptides containing non-protein amino acids. Furthermore, we proved that large and bulky amino acid residues such as Ac_8c could be successfully incorporated in the preptin sequence using manual solid-phase peptide synthesis. Stability and insulin secretion studies using the preptin analogues indicate that modification of Phe 21 using the non-protein amino acid substitutes investigated does not affect its insulin secretory activity but does make it resistant to hydrolysis by chymotrypsin at that position. Our results also show for the first time that at low micromolar concentrations, preptin (and analogues) acts to stimulate insulin secretion in both the presence and absence of glucose. We further observed that of the substitutes investigated, D-Phe and D-Hphe consistently performed the best with regard to insulin secretion, while the effects of 3-abz-OH and Ac_8c were not significantly different from controls, indicating that the observed propensity for Ac_8c to form helices does not translate into improved bioactivity in this family of peptides. While the protease that hydrolyses preptin at position Phe 21 *in vivo* has not been identified, we are encouraged by these findings and will pursue *in vivo* stability studies to confirm these observations.

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