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Synthesis of truncated analogues of preptin-(1–16), and investigation of their ability to stimulate osteoblast proliferation

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ABSTRACT

Preptin, a 34-amino acid residue peptide hormone is co-secreted with insulin from the β-pancreatic cells and is active in fuel metabolism. We have previously established that a shorter fragment of preptin, namely preptin-(1–16), stimulates bone growth by proliferation and increasing the survival rate of osteoblasts. This was demonstrated in both in vitro and in vivo models. These findings suggest that preptin-(1–16) could play an important role in the anabolic therapy of osteoporosis. However, due to the large size of the peptide it is not an ideal therapeutic agent. The aim of this study was to identify the shortest preptin analogue that retains or even increases the bone anabolic activity as compared to the parent preptin-(1–16) peptide. Truncations were made in a methodical manner from both the N-terminus and the C-terminus of the peptide, and the effect of these deletions on the resulting biological activity was assessed. In order to improve the enzymatic stability of the shortest yet active analogue identified, ruthenium-catalysed ring closing metathesis was used to generate a macrocyclic peptide using allylglycine residues as handles for ring formation. We have successfully identified a short 8-amino acid preptin (1–8) fragment that retains an anabolic effect on the proliferation of primary rat osteoblasts and enhances bone nodule formation. Preptin (1–8) is a useful lead compound for the development of orally active therapeutics for the treatment of osteoporosis.

1. Introduction

The bone disease osteoporosis (literally meaning 'porous bone'), is characterized by reduced bone mass and disruption of bone microarchitecture, leading to weakened bones and increased risk of fractures.[1] A major health issue worldwide, osteoporosis affects millions of people globally, predominantly postmenopausal women. Estimates suggest one in three women and one in five men over the age of fifty worldwide will sustain an osteoporotic fracture, leading to a significant burden on both the individual and society.[1] Partly due to the increased longevity of the population, and rise in expectation of increased physical activity in the elderly, the number of fractures is expected to rise in the near future.[2]

Healthy bone is maintained throughout life by a well-coordinated balance between bone-forming cells called osteoblasts and bone-resorbing cells called osteoclasts. During osteoporosis, an imbalance between bone resorption and bone formation leads to the development of fragile bone tissue. Most current osteoporotic therapies are anti-resorptive, inhibiting bone loss by reducing osteoclast activity.[3] Unfortunately, anti-resorptive therapy cannot restore bone mass or bone structure already lost due to increased bone remodelling. Therapies acting on osteoblasts are therefore highly attractive, but the only such treatment currently in use is human parathyroid hormone (PTH) and its N-terminal fragment, teriparatide (PTH 1–34).[2] Unfortunately, PTH therapy is limited due to its high cost, the need for daily subcutaneous injections, and side effects including hypercalcemia and a possible increased risk of osteosarcoma.[4] Considerable demand exists for a new anabolic drug to treat osteoporosis.

Preptin is a 34-residue polypeptide hormone secreted from the pancreatic β-cells that corresponds to the Asp(69)-Leu(102) fragment of proIGF-II E (Fig. 1).[5] It was isolated from cultured pancreatic β-cells, along with insulin and amylin. All three hormones are involved in glucose metabolism, and preptin may

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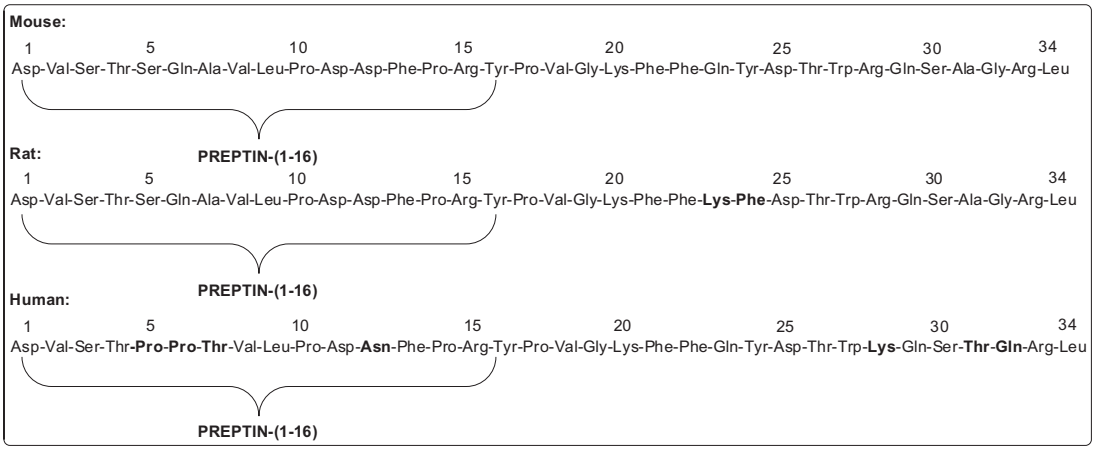


Figure 1. Comparison of primary structures of mouse, rat, and human preptin.

be a physiological amplifier of glucose-mediated insulin secretion.[6] Rat preptin-(1–34) and analogues in which Phe-21 was substituted by various unnatural amino acid residues, stimulate insulin secretion in the presence and absence of glucose.[7]

We have recently demonstrated a stimulatory effect of rat preptin on bone growth[6] and have established that preptin-(1–34) acts on osteoblast-like cells to stimulate proliferation, differentiation and survival in vitro and in vivo.[6] It was observed that proliferative effect of preptin is mediated through a G protein-coupled receptor activating G_i-dependent phosphorylation of p42/44 MAP kinases.[6] Interestingly, a human condition of high bone mass phenotype has been observed when increased circulating levels of a pro-IGF-II peptide complexed with IGF-binding protein-2 occur in patients with chronic hepatitis C infection. Additionally, Li et al.[8] have recently shown that osteoporotic men have lower circulating levels of preptin than healthy individuals. These results clearly demonstrate the osteogenic nature of preptin in humans. However, little is known about the mechanism of action of preptin and its influence on bone metabolism and the preptin receptor is still unknown. Liu et al.[9] have shown that connective tissue growth factor (CTGF) mediates preptin-induced proliferation and differentiation of human osteoblasts. CTGF plays an important role in skeletal development stimulating osteoblastic cells for proliferation and differentiation. These findings suggest that preptin might be a very promising new pharmaceutical lead for the anabolic treatment of osteoporosis.

We have observed that a shorter rat preptin fragment, namely preptin-(1–16), is still anabolic to osteoblasts in both in vitro and in vivo studies, and unlike full-length preptin has no activity in the pancreas thus it does not affect carbohydrate metabolism (Fig. 1).[10] This smaller peptide is a more attractive candidate for pharmaceutical development and can be used as a model peptide for the creation of simpler but more orally active analogues.

To the best of our knowledge no reports have been documented in the literature on the study of preptin and preptin analogues as drug candidates to treat osteoporosis. Therefore, the aim of this study was to investigate whether N- and C-modified analogues of rat preptin-(1–16) and its truncated analogues, retain or increase anabolic activity on bone growth compared to the parent 16-amino acid residue peptide. Due to our on-going interest in the design and synthesis of peptidomimetics of biologically active peptides we were also interested to see if the lead peptide identified during the study could be used to afford more stable and thus more attractive pharmaceutical candidates.[11–16]

We herein report the synthesis of preptin-(1–16) (**1**), its *N*-acetylated, and *C*-amidated analogues (**2**–**4**) and a family of truncated analogues containing modifications of the N- and C-termini (**5**–**28**). A ring closing metathesis (RCM) technique was used to synthesize a hydrocarbon linked cyclic peptide **29** in the search of an enzymatically more stable therapeutic to treat osteoporosis (Fig. 2).[17,18] All analogues with the exception of **11** were evaluated for their proliferative activity in vitro using primary rat osteoblasts. The lead compounds identified (**9**, and **20**) were further tested for their ability to form bone nodules. This is the first study that enables identification of the shortest biologically active preptin-(1–16)-derived peptide with potential to treat osteoporosis as an agent delivered orally.

2. Results and discussion

2.1. Synthesis of preptin-(1–16) (1), *N*-acetylated preptin-(1–16) (2), *C*-amidated preptin-(1–16) (3), and *N*-acetylated and *C*-amidated preptin-(1–16) (4)

As depicted in Scheme 1 Fmoc Solid Phase Peptide Synthesis (SPPS) was employed for the preparation of rat preptin-(1–16) (**1**), *N*-acetylated preptin-(1–16) (**2**), *C*-amidated preptin-(1–16) (**3**), and *N*-acetylated and *C*-amidated preptin-(1–16) (**4**). In order to obtain a C-terminal acid for peptides **1** and **2**, the growing peptide chain was attached to the solid support via 4-(hydroxymethyl)phenoxypropionic acid linker (HMPP). In order to afford the C-terminal amide in preptin-(1–16) analogues **3** and **4**, Rink amide linker was employed to anchor the growing peptide chain to the resin.

Our initial focus was to develop reliable synthetic conditions that allow the rapid generation of a library of analogues of the preptin-(1–16) peptide. Use of microwave irradiation reduces the times for SPPS affording improved synthetic yields[19] hence it was adopted in the present work. Preptin-(1–16) contains three aspartic acid residues (Asp-1, Asp-11, and Asp-12) in its sequence that can potentially undergo aspartimide formation when using piperidine for N^{α}-Fmoc protecting group removal under microwave conditions.[20,21] It was therefore decided to use a milder deprotection base, namely piperazine (5% in DMF).[19,21,22] However, formation of aspartimide by-products was not reported during the synthesis of preptin-(1–34) and its analogues when the synthesis was performed at room temperature using 20% piperidine in DMF.[7]

HBTU is one of the most commonly used coupling reagents[23] and we anticipated its use for the current study.[24,25] Although coupling of Arg-15 was challenging when HBTU was employed as the coupling reagent for the synthesis of preptin-(1–34) for the synthesis of shorter fragments of preptin using microwave-enhanced

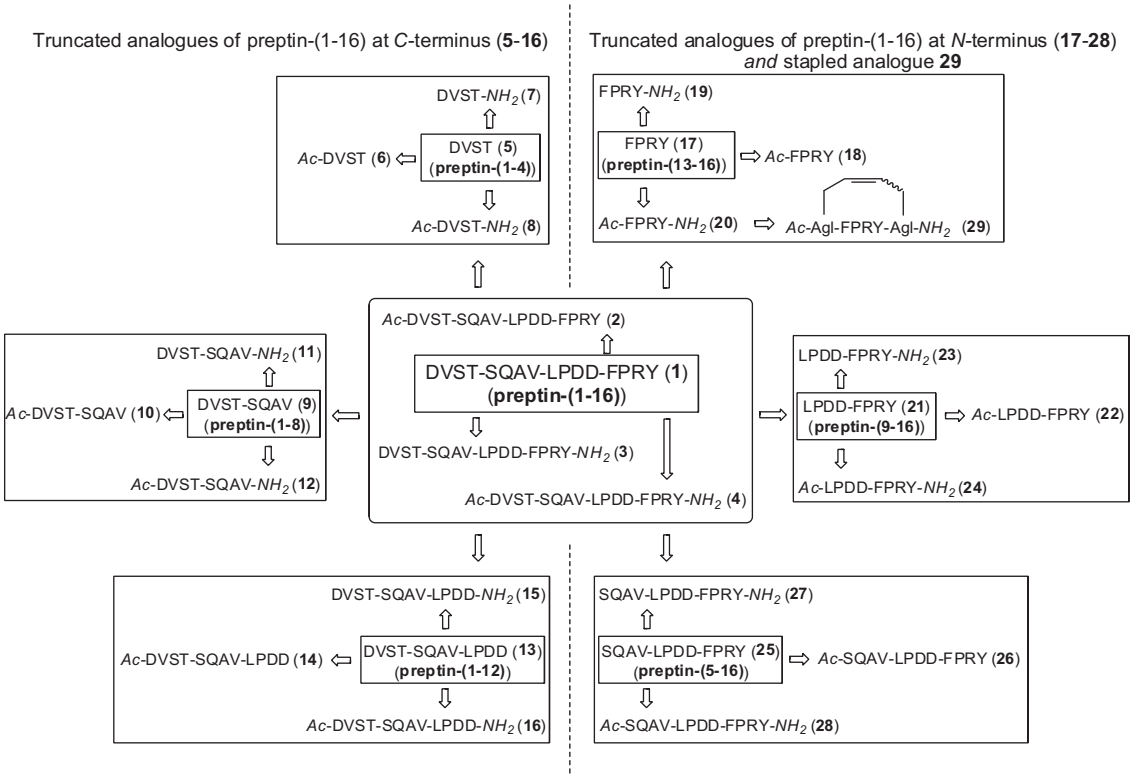
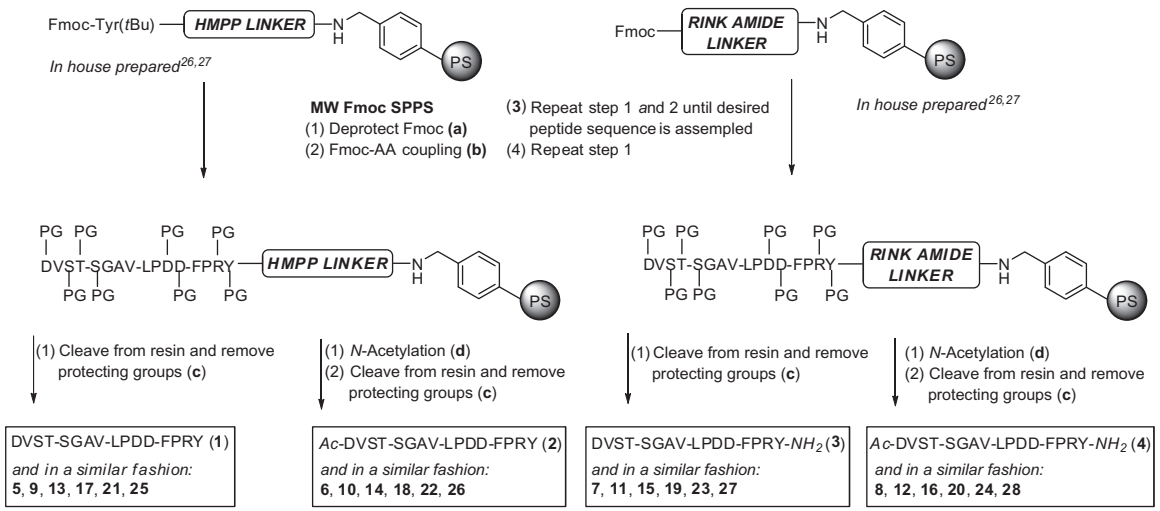


Figure 2. Preptin-(1–16) (**1**) and its analogues (**2**–**29**) synthesised during the study.



Reagents and conditions:
(a) 5% piperazine, DMF, (compounds **1**-**4**) and 5% piperazine, 0.1 M 6-Cl-HOBt, DMF, (compounds **5**-**28**), 62 W, 75 °C, 1 x 0.5 min + 1 x 3 min;
(b) HBTU, iPr$_2$EtN, DMF, 25 W, 75 °C, 5 min (all Fmoc-AAexcept Fmoc-Arg(Pbf)) and *(i)* 30 min, rt *(ii)* 25W, 72 °C, 5 min (Fmoc-Arg(Pbf));
(c) TFA, iPr$_3$SiH, DODT, H_2O, rt, 2 h; **(d)** 20% Ac_2O, DMF, rt, 2 x 20 min.

Scheme 1. Synthesis of preptin-(1–16) (**1**), its analogues **2**–**4** and truncated analogues at the C-terminus (**5**–**16**) and at the N-terminus (**17**–**28**).

conditions it was anticipated that HBTU/iPr$_2$EtN would be a suitable coupling reagent.

'In house' prepared aminomethyl polystyrene resin (AM-PS) functionalised with either an N^{α}-Fmoc-protected tyrosine residue attached to the HMPP linker (for peptides **1** and **2**) or Rink amide linker (for analogue **3** and **4**)[26,27] was used for SPPS that was carried out using a CEM Liberty 1 peptide synthesiser. The final step for the synthesis of native preptin-(1–16) (**1**) was TFA mediated cleavage of the resin-bound peptide **1** with concomitant removal of side chain protecting groups which was performed at room temperature. Pleasingly, the expected product **1** was afforded as the major product (as measured by RP-HPLC at 210 nm) and contained only one aspartimide (18 Da less) as a minor impurity (22%). The site of aspartimide formation was not determined.

Initial N-acetylation of Fmoc-deprotected N^{α} amino group of Asp-1 of resin-bound peptide **2** (Scheme 1) was required before final treatment with TFA to afford crude *N*-acetylated preptin-(1–16) analogue (**2**). RP-HPLC analysis of the crude mixture

revealed that the expected product **2** was present as the major product, and similar to the synthesis of the parent peptide **1** it only contained one aspartimide as a minor by-product (23%). Table 1 summarises RP-HPLC and MS analysis for the peptides **1**–**4** (Supporting information). These results demonstrated that use of a milder base (piperazine) than piperidine under microwave conditions was not sufficient to suppress aspartimide formation.

Subsequent synthesis of *C*-amidated preptin-(1–16) (**3**), and *N*-acetylated and *C*-amidated preptin-(1–16) (**4**) using Rink amide linker following the same synthetic route as for **1** and **2** afforded crude peptides **3** and **4** in good purity (55%, and 63%, respectively) contaminated with an aspartimide by-product (26%, Scheme 1). The presence of the bulkier Rink amide linker used for the synthesis of *C*-amidated analogues (**3**), and (**4**) had no effect on aspartimide formation compared to the HMPP linker used for the synthesis of preptin-(1–16) (**1**) and *N*-acetylated preptin-(1–16) (**2**). The rate of aspartimide formation depends on the nature of the neighbouring residues to Asp on both the N-terminus and C-terminus and we were interested to see if the type of the linker influenced formation of this by-product.[20]

Pleasingly, subsequent RP-HPLC purification enabled separation of the desired products **1**–**4** from the aspartimide by-product to afford peptides **1**–**4** in excellent >98% purity (Table 1, Supporting information). However, further optimisation of the Fmoc deprotection conditions may be required in the future. It is anticipated that formation of the undesired aspartimide by-product can be further prevented by addition of an acid additive (hydroxybenzotriazole-based) to the piperazine-based deprotection mixture as previously described.[19,21] Given that we successfully separated the desired product in quantities that were sufficient for subsequent biological assays, further optimisation of the synthesis of **1**–**4** was not undertaken in this study.

2.2. Synthesis of C-terminus truncated analogues of preptin-(1–16) (5–16)

In order to identify the minimum length of the peptide that still retains or increases the activity of the parent preptin-(1–16) **1**, C-terminal truncated analogues were initially prepared. Thus, synthesis of preptin-(1–4) (**5**), preptin-(1–8) (**9**), preptin-(1–12) (**13**), their derivatives acetylated at the N-terminus (**6**, **10**, **14**), amidated at the C-terminus (**7**, **11**, **15**) and, both *N*-amidated and *C*-acetylated (**8**, **12**, **16**), was undertaken following the synthetic method previously used for peptides **1**–**4** (Scheme 1). Due to the observed formation of an aspartimide by-product during the synthesis of preptin-(1–16) (**1**) and its analogues **2**–**4**, 5% piperazine solution in DMF with 0.1 M 6-Cl-HOBt as additive was used to effect N^{α}-Fmoc-protecting group removal thereby minimising potential aspartimide formation.[21]

Once the desired peptide sequences for analogues **5**, **9**, **13**, and their *C*-amidated analogues (**7**, **11**, and **15**, respectively) were assembled, peptides were detached from resin (TFA) affording peptides with crude purity greater than 80%. The purity of the crude analogue DVST-NH_2 (**7**) could not be determined due to difficulties experienced in retaining peptide **7** on the analytical column under the conditions used for RP-HPLC. However, ESI-MS analysis of crude analogue **7** confirmed that the desired product was formed as the major product (*m*/*z* 420.2) $[M+H]^+$ requires 420.44. Pleasingly, no presence of an aspartimide by-product was detected. A summary of the chromatographic and mass spectrometry data for compounds (**5**–**16**) is given in Table 1 (Supporting information).

Fully assembled, resin-bound and N^{α}-Fmoc-deprotected truncated analogues (**6**, **8**, **10**, **12**, **14**, and **16**) were initially treated with acetic anhydride and the resin was subsequently cleaved using TFA to afford crude *N*-acetylated (**6**, **10**, and **14**), and both, *N*-acetylated and *C*-amidated (**8**, **12**, and **16**) shorter analogues of preptin-(1–16) (**1**) with purities ranging from 72% (analogue **12**) to 97% (analogue **6**). Similar to DVST-NH_2 (**7**), its *N*-acetylated derivative **8** proved difficult to retain on the analytical RP-HPLC column thus the purity of crude peptide **8** was not calculated and the presence of the desired product **8** was confirmed using ESI-MS analysis (*m*/*z* 462.2) $[M+H]^+$ requires 461.46.

Peptides **5**–**16** were subjected to final RP-HPLC purification to afford the C-terminus truncated analogues of preptin-(1–16) (**1**) in excellent purity ranging from 93% to 99% (Table 1, Supporting information) ready for subsequent biological evaluation. It was unexpected to find that preptin-(1–8)-NH_2 (**11**) was poorly soluble in solvents used for the subsequent bioassay (methanol, DMSO, H_2O) therefore it was decided to withdraw analogue **11** from further study. The reason for insolubility of **11** is unknown.

2.3. Synthesis of N-terminus truncated analogues of preptin-(1–16) (17–28)

Initial synthesis of analogues of preptin-(1–16) truncated at the C-terminus afforded eleven shorter peptides (**5**–**10**, and **12**–**16**) that were assessed for their anabolic bone activity in vitro. Subsequent preparation of analogues that were truncated at the N-terminus, namely preptin-(13–16) (**17**), preptin-(9–16) (**21**), preptin-(5–16) (**25**), their *N*-acetylated analogues (**18**, **22**, **26**), as well as *C*-amidated variants (**19**, **23**, **27**), and both *N*-acetylated and *C*-amidated analogues (**20**, **24**, **28**) was next undertaken (Fig. 2).

Microwave-enhanced Fmoc SPPS using the synthetic conditions summarised in Scheme 1 successfully delivered the desired products **17**, **21**, **25** and their *C*-amidated derivatives (**19**, **23**, **27**), respectively. The crude peptides were obtained with purities varying from 84% to 94% (Table 1, Supporting information). Resin-bound and N^{α}-terminally deprotected peptides **18**, **20**, **22**, **24**, **26, 28** were treated with acetic anhydride before TFA mediated resin cleavage to afford *N*-acetylated preptin-(13–16) (**18**), preptin-(9–16) (**22**), preptin-(5–16) (**26**), and their *C*-amidated analogues (**20**, **24**, and **28**), respectively, with crude purities >81% (Table 1, Supporting information). Subsequent RP-HPLC purification of truncated N-terminus analogues of preptin-(1–16) gave products **17**–**28** with purities >98% with the exception of Ac-SQAV-LPDD-FPRY (**26**) and Ac-SQAV-LPDD-FPRY-NH_2 (**28**) (96% and 94%, respectively) ready for subsequent biological assessment.

2.4. In vitro proliferation effect of synthesised analogues on primary rat osteoblasts

Preptin-(1–16) (**1**) and synthetic analogues **2**–**10** and **12**–**28** were assessed for their proliferation of bone activity in vitro using cultures of foetal rat osteoblasts as previously described.[28] The effects of the compounds tested were evaluated by measuring the $[^3H]$-thymidine incorporation levels over the last 6 h of the incubation period (24 h). Pleasingly, three out of the twenty six preptin-(1–16) analogues tested, namely, Ac-preptin-(1–16)-NH_2 (**4**), preptin-(1–8) (**9**), and Ac-preptin-(13–16)-NH_2 (**20**), together with the intact preptin-(1–16) (**1**) showed a significant increase in thymidine incorporation levels (a 5% significance level was used throughout) (Fig. 3). The rest of the peptides studied (**2**, **3**, **5**–**8**, **10**, **12**–**19**, **21**–**28**) had no significant effect on osteoblast proliferation (data not shown).

Ac-preptin-(1–16)-NH_2 (**4**) has the same length as native preptin-(1–16) (**1**), thus our focus was only directed at the smaller analogues **9** and **20**. Interestingly, these two smaller peptides are both derived from two distinct preptin-(1–16) sites, N-terminal (analogue **9**), and C-terminal (analogue **20**), suggesting that the internal region of the preptin-(1–16) (fragment 9–12) is not important for bone activity. These much shorter peptide analogues are attractive

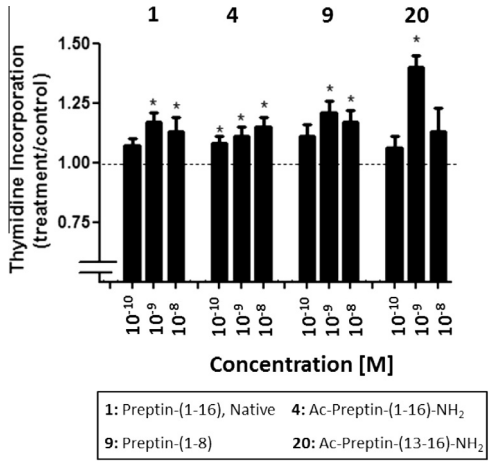


Figure 3. Osteoblast activity of the preptin-(1–16) (**1**) and analogues tested showing the significance level (**4**, **9**, and **20**). Data are expressed as a ratio of treatment to control, mean ± SEM; *significantly different from control (p <0.05).

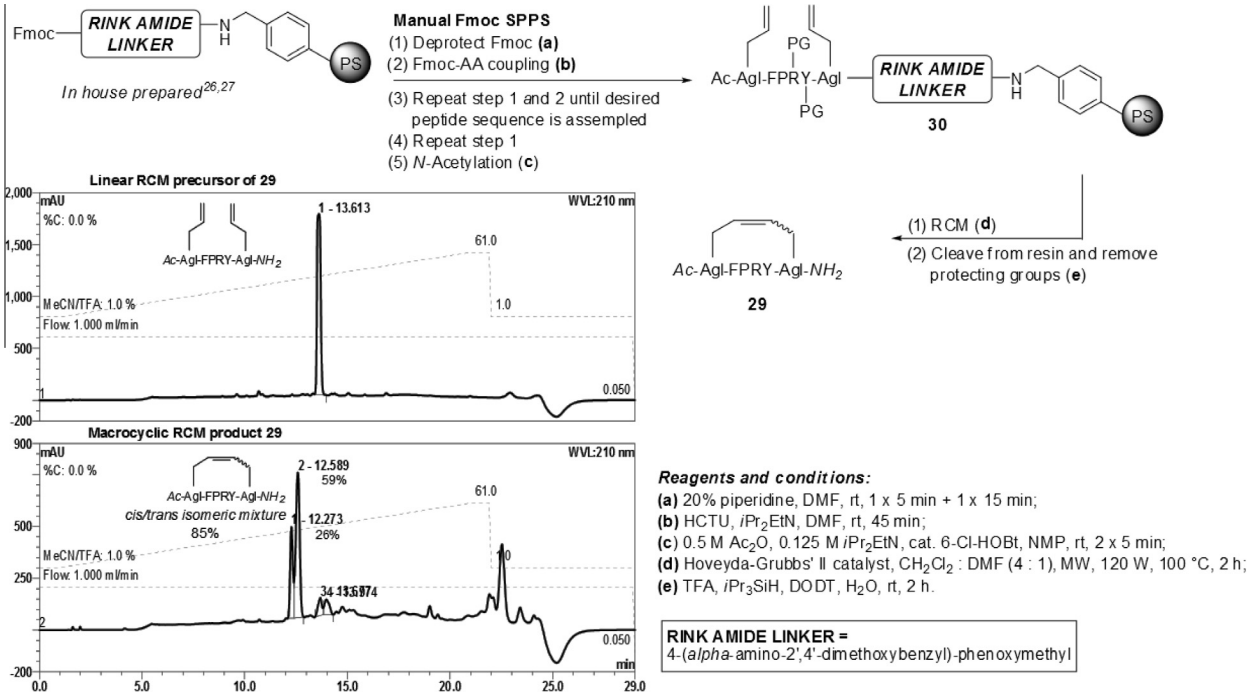
targets for further drug development as they have greater potential to afford orally available agents than preptin-(1–16). However, the poor pharmacokinetic profile of these natural peptides requires further chemical modifications to generate more stable and more bioavailable compounds. We therefore decided to initially attempt to optimise the structure of the shortest active preptin-(1–16) fragment, Ac-preptin-(13–16)-NH_2 (**20**).

2.5. Synthesis and osteoblast proliferation activity of macrocyclic analogue 29

Bioactive peptides comprising natural amino acids in their sequence exhibit a high degree of flexibility and are susceptible to protease degradation which hampers their use as therapeutics.[29] These major obstacles make them unsuitable for drug development. A peptidomimetic toolbox offers many types of structural modifications to peptides in order to deliver compounds with improved pharmacokinetic profile.[29] Peptide cyclization using ring closing metathesis generates stable carbon–carbon bonds and is often used in the drug discovery area to increase metabolic stability, improve peptide activity, and stabilise peptide conformation.[30–32]

A RCM technique has been successfully used in our laboratory to afford biologically active analogues of amylin-(1–8) therefore, we decided to use this technique in the current study.[12] Our focus was to synthesise a macrocyclic analogue of the shortest, 4-amino acid active peptide, Ac-preptin-(13–16)-NH_2 (**20**). To minimise disruption to the primary sequence of this short analogue it was decided to introduce allylglycine (Agl) residues required for subsequent RCM at both the N- and the C-terminus of the peptide, acetylate the N^{α}-terminus and amidate C-terminus. Attention thus focused on the synthesis of the RCM analogue **29** (Scheme 2).

With this idea in mind, the synthesis of the resin-bound linear RCM precursor **30** was undertaken starting from in house prepared aminomethyl polystyrene resin modified with Rink amide linker using manual Fmoc SPPS at room temperature.[26,27] 20% piperidine in DMF was used to remove the N^{α}-Fmoc protecting group, and amino acid couplings were performed using HCTU with iPr$_2$EtN as a base. Successive steps of Fmoc deprotection and couplings of N^{α}-Fmoc-protected amino acids followed by final N-acetylation of the free N^{α}-amino group of N-terminal allylglycine (acetic anhydride in NMP) afforded Ac-Agl-Phe-Pro-Arg(Pbf)-Tyr(tBu)-Agl-Rink amide-PS peptidyl resin **30**. Subsequent TFA mediated cleavage of an aliquot of resin **30** followed by LCMS analysis confirmed the presence of the desired RCM precursor ready for subsequent macrocyclization. Hoveyda–Grubbs' II catalyst (30 mol % of catalyst, CH_2Cl_2/DMF (4:1) MW, 100 °C, 2 h) was used to effect the on-resin ring closing metathesis reaction, followed by removal of the macrocyclic peptide from the resin (TFA/iPr$_3$SiH/H_2O/DODT).[12] Pleasingly, good conversion to the desired RCM product as an inseparable mixture of *cis* and *trans* isomers was observed as revealed by RP-HPLC analysis (Scheme 2, $R_{tcis\ or\ trans}$ 12.27 min, 26%, $R_{tcis\ or\ trans}$ 12.59 min, 59%). Further purification of the crude



Scheme 2. Synthesis of RCM macrocyclic analogue of preptin-(1–16) (**29**).

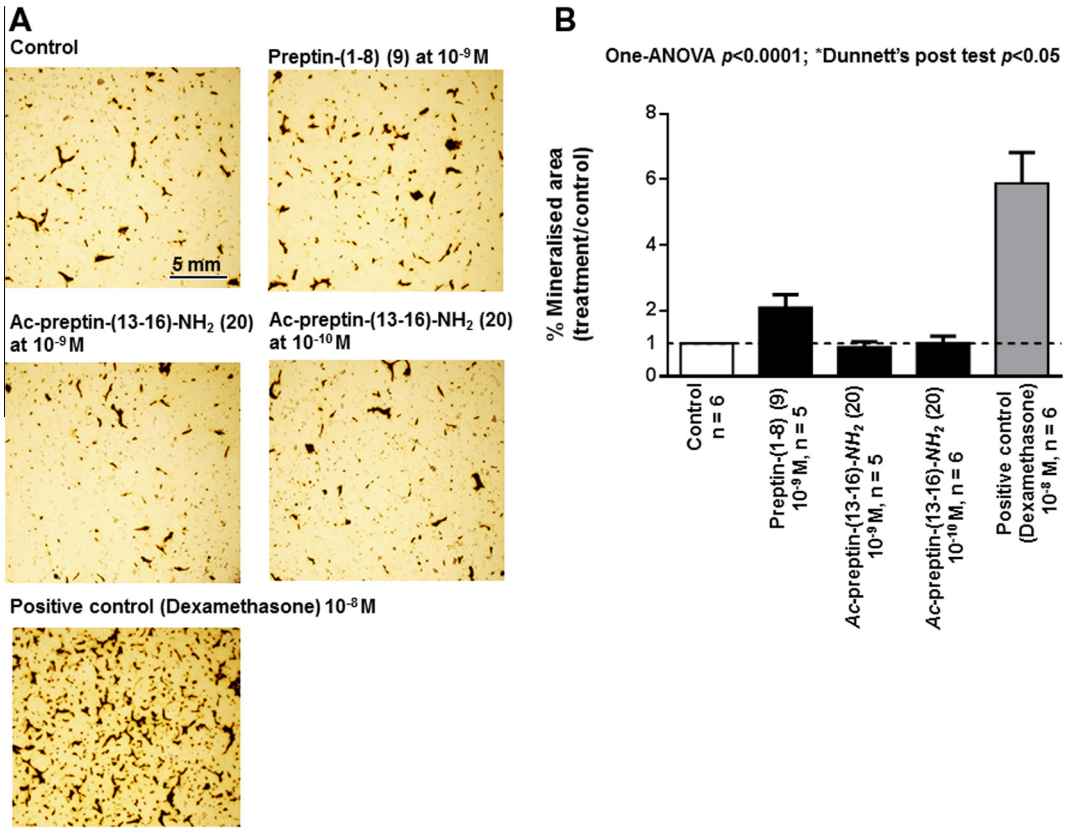


Figure 4. Effect of preptin-(1–8) (**9**) and Ac-preptin-(13–16)-NH_2 (**20**) on formation of bone nodules in primary cultures of rat osteoblast cells over a period of 3 weeks. Cultures were stained for mineral using Von Kossa stain, and area of mineralized bone nodules were quantified. A shows nodule formation and B shows % of mineralised area.

product by RP-HPLC was performed affording peptide **29**, as a mixture of *cis* and *trans* isomers ready for biological analysis.

Macrocyclic analogue **29** was subsequently tested for its ability to stimulate the proliferative activity of primary rat osteoblasts. Similar to previous analysis of preptin-(1–16) (**1**) and its analogues **2**–**10**, and **12**–**28**, [^{3}H]-thymidine incorporation was measured and compared to control. It was disappointing to see however, that no significant activity to stimulate osteoblasts was observed. It is difficult to account for this lack of activity. The altered primary structure of **29** with the presence of non-natural allylglycine residues, and the more constrained peptide conformation introduced by the macrocyclic ring in RCM analogue **29** compared to the linear peptide **20** could account for the lack of activity. Additionally, the fact that the sequence of both linear analogue **20**, and macrocyclic analogue **29** comprise only four out of the sixteen amino acids in preptin-(1–16) may also be a contributing factor. Interestingly, proliferation activity was observed for the truncated, natural Ac-preptin-(13–16)-NH_2 (**20**) but no osteoblast stimulation effect was observed for the macrocyclic peptide **29**. We therefore decided to further investigate the biological activity of the linear analogues, preptin-(1–8) (**9**) and Ac-preptin-(13–16)-NH_2 (**20**) using a bone nodule formation assay, before further structural modifications were undertaken. NMR analysis of macrocyclic analogue **29** to establish the *cis*/*trans* isomeric ratio was not performed due to the lack of activity observed in the osteoblast proliferation assay.

2.6. Effect of preptin-(1–8) (9) and Ac-preptin-(13–16)-NH_2 (20) on formation of bone nodules in primary rat osteoblast cells

In order to further demonstrate the anabolic activity of both the lead peptides identified (**9** and **20**), the effect of both preptin-(1–8) (**9**), and Ac-preptin-(13–16)-NH_2 (**20**), on primary rat osteoblasts differentiation was studied using a bone nodule formation assay with dexamethasone used as a positive control. The appropriate peptides (**9**, or **20**) were added to the confluent cells suspended in media supplemented with osteoblast differentiation substances (L-ascorbic acid-2-phosphate and β-glycerophosphate). After 21 days, the cells were fixed, stained by Von Kossa stain, and the mineralized areas were quantified using Bioquant image analysis software. The process of bone nodule formation relies on the activity of differentiated osteoblasts to deposit and mineralise bone matrix. We were pleased to see that preptin-(1–8) (**9**) significantly stimulated formation of bone nodules and was able to increase the percentage of mineralised area in primary rat osteoblasts at 10^{-9} M concentration. However, the shorter preptin-(1–16) analogue, Ac-preptin-(13–16)-NH_2 (**20**) did not demonstrate a significant effect on bone nodule formation and mineralisation at concentration of 10^{-9} M and 10^{-10} M (Fig. 4). These results have further established the importance of the N-terminal region of native preptin-(1–16) that is most likely responsible for the observed anabolic effect on osteoblasts. Future structure-activity optimisation studies will therefore be focused on the key preptin-(1–8) fragment.

3. Conclusions

Our goal to identify the shortest analogues of the biologically active native preptin-(1–16) peptide that retain bone-forming activity was informed by the successful synthesis of a library of twenty eight analogues of preptin-(1–16) and their in vitro assessment on primary rat osteoblasts. We have established the importance of the N-terminal region of preptin-(1–16) to stimulate osteoblasts. This is the first study of this kind that has enabled the successful identification of the shortest preptin analogue (**9**), namely preptin-(1–8), that retained bone activity in both

proliferative and differentiation studies thus proving that its anabolic action increases the number of bone forming cells as well as the function of the cells. This 8-amino acid peptide is much smaller than the parent 16-mer molecule and is therefore an interesting target to develop new chemical entities for the oral treatment of osteoporosis. The design and synthesis of chemically modified preptin-(1–8) (**9**) analogues to further improve activity and/or stability is currently being undertaken in our lab and the results of this study will be reported in due course.

4. General methods

4.1. Chemistry

4.1.1. Materials

All reagents were purchased as reagent grade and used without further purification. *O*-(Benzotriazol-1-yl)-*N*,*N*,*N*′,*N*″-tetramethyluronium hexafluorophosphate (HBTU), *O*-(6-chlorobenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HCTU), 4-[(*R*,*S*)-α-[1-(9*H*-floren-9-yl)]methoxycarbonylamino]-2,4-dimethoxy]phenoxyacetic acid (Rink linker), and Fmoc-amino acids were purchased from GL Biochem (Shanghai, China). Fmoc-amino acids were supplied with the following side chain protection: Fmoc-Asp(O*t*Bu), Fmoc-Ser(*t*Bu), Fmoc-Thr(*t*Bu), Fmoc-Arg(Pbf). Piperazine, *N*,*N*-diisopropylethylamine (*i*PrNEt), ***N***,***N***′-diisopropylcarbodiimide (DIC), 3,6-dioxa-1,8-octane-dithiol (DODT), formic acid, 1-methyl-2-pyrrolidinone (NMP), 1,3-bis-(2,4,6-trimethylphenyl)-2-imidazolidinylidene)dichloro(*o*-isopropoxyphenylmethylene)ruthenium (Hoveyda–Grubbs' II catalyst), and triisopropylsilane (iPr$_3$SiH) were purchased from Sigma–Aldrich (Sydney, Australia). *N*,*N*-Dimethylformamide (DMF), acetonitrile (ACN), and hydrochloric acid (HCl), were supplied from Scharlau (Barcelona, Spain). Dichloromethane (CH_2Cl_2) was purchased from ECP Limited. Trifluoroacetic acid (TFA) was purchased from Halocarbon (River Edge, New Jersey), 6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt) was purchased from Aapptec (Louisville, Kentucky), and Fmoc-AA-$OCH_2PhOCH_2CH_2CO_2H$ (Fmoc-AA-HMPP) were purchased from PolyPeptide Group (Strasbourg, France). Dimethyl sulfoxide (DMSO) was purchased from Romil Ltd (Cambridge, United Kingdom). Aminomethyl polystyrene resin was synthesised 'in house' as previously described.[26,27]

4.1.2. Peptide synthesis, purification and analysis

For the synthesis of peptides affording a C-terminal acid (**1**, **2**, **5**, **6**, **9**, **10**, **13**, **14**, **17**, **18**, **21**, **22**, **25**, **26**), AM-PS resin was initially swollen in CH_2Cl_2 (30 min) and subsequently reacted with Fmoc-AA-$OCH_2PhOCH_2CH_2CO_2H$ (2.0 equiv), and DIC (2.0 equiv) in CH_2Cl_2 (2.0 mL) for 2 h at room temperature.[27] The Kaiser test was negative.[33] For the synthesis of peptides containing C-terminal amide (**3**, **4**, **7**, **8**, **11**, **12**, **15**, **16**, **19**, **20**, **23**, **24**, **27**, **28**, **29**), AM-PS resin was initially swollen in DMF (30 min) and subsequently reacted with Rink linker (5.0 equiv), DIC (5.0 equiv), and 6-Cl-HOBt (5.0 equiv) in DMF (2.0 mL) for 2 h at room temperature.[27] The Kaiser test was negative.[33] Fmoc SPPS was then performed on a Liberty 1 Microwave Peptide Synthesiser (CEM Corporation, Mathews, NC) on a 0.1 mmol scale using the Fmoc/*t*Bu strategy. All amino acid couplings were performed as single coupling cycles, with the exception of Fmoc-Arg(Pbf) where a double coupling cycle was performed as part of a synthetic protocol recommended by CEM Microwave Technology. All Fmoc-AA couplings were performed using Fmoc-AA (5.0 equiv, 0.2 M), HBTU (4.5 equiv, 0.45 M), and *i*PrNEt (10 equiv, 2 M) in DMF, for 5 min, at 25 W and maximum temperature of 75 °C, except Fmoc-Arg(Pbf) that was initially coupled for 25 min at room temperature which was followed by the second coupling for 3 min, at 25 W and maximum temperature of 72 °C. Fmoc protecting group was removed using 5% piperazine in DMF (compounds **1**–**4**) and 5% piperazine with 0.1 M 6-Cl-HOBt in DMF (compounds **5**–**28**). A 30 s deprotection cycle was followed by a second deprotection for 3 min at 62 W and maximum temperature of 75 °C.

Acetylation was performed by treatment of the resin with acetic anhydride (20% in DMF) at room temperature (2 × 20 min), or by using acetic anhydride (0.5 M in NMP), iPr$_2$EtN (0.125 M in NMP) and a catalytic quantity of HOBt in NMP (room temperature, 2 × 5 min).

A fritted glass reaction vessel was used for the manual synthesis of **29** (0.25 mmol scale). The Fmoc protecting group was deprotected with 20% piperidine solution in DMF (1 × 5 min, 1 × 15 min). The resin was washed with DMF (5 × 5 mL), and Fmoc-AA coupling was performed. N^{α}-Fmoc-protected amino acid (4.0 equiv) was dissolved in DMF, HCTU (3.8 equiv) was added and mixture shaken until dissolved. The solution was transferred to the reaction vessel and shaken for 2 min, followed by the addition of iPr$_2$EtN (8.0 equiv). The mixture was shaken for 45 min, filtered, and washed with DMF (3 × 5 mL).

The resulting peptides were cleaved from the resin with simultaneous side chain protecting group removal by treatment with TFA/iPr$_3$SiH/H_2O/DODT (v/v/v/v; 94/1/2.5/2.5), for 2 h at room temperature. The crude peptides were precipitated and triturated with cold diethyl ether, isolated (centrifugation), dissolved in 20% acetonitrile (aq) containing 0.1% TFA and lyophilized.

Analytical reverse phase high-performance liquid chromatography (RP-HPLC) was performed using either a Dionex P680 or Dionex Ultimate U3000 system (flow rate of 1 mL/min), using Waters XTerra® column (MS C_{18}, 150 mm × 4.6 mm; 5 μm) using gradient systems as indicated in the Supporting information.

The solvent system used was A (0.1% TFA in H_2O) and B (0.1% TFA in acetonitrile) with detection at 210 nm, 254 nm, and 280 nm. The ratio of products was determined by integration of spectra recorded at 210 nm. A Hewlett Packard (HP) 1100MSD mass spectrometer using ESI in the positive mode spectrometer was used for ESI-MS analysis (positive mode). Peptide purification was performed using a Waters 600E system using a semipreparative Phenomenex Gemini C_{18}, 250 mm × 10 mm; 5 μm column or Phenomenex Luna C_8, 250 mm × 10 mm; 5 μm column. Gradient systems were adjusted according to the elution profiles and peak profiles obtained from the analytical RP-HPLC chromatograms. Fractions were collected, analysed by either RP-HPLC or ESI-MS, pooled and lyophilised 3 times from 10 mM aq HCl.

4.2. Biology

4.2.1. Bone growth activity assays (proliferation)

Osteoblasts were isolated from 20-day fetal rat calvariae, as previously described.[28] Briefly, calvariae were excised and the frontal and parietal bones, free of suture and periosteal tissue, were collected. The calvariae were sequentially digested using collagenase (Sigma) and the cells from third and fourth digests were collected, pooled and washed. Cells were grown in T75 flasks in 10% FBS/Dulbecco's modified eagle medium (DMEM)(Invitrogen) and 5 μg/mL L-ascorbic acid 2-phosphate (Sigma) for 2 days and then changed to 10% FBS/MEM (Invitrogen)/ 5 μg/mL L-ascorbic acid 2-phosphate and grown to 90% confluency. Cells were then seeded into 24 well plates at 2.5×10^4 cells/well in 5% FBS/MEM 5 μg/mL L-ascorbic acid 2-phosphate for 24 h. Cells were growth-arrested in 0.1% bovine serum albumin (BSA) (ICP, Auckland, New Zealand)/5 μg/mL L-ascorbic acid 2-phosphate for 24 h. Cells were pulsed with [^{3}H] thymidine 6 h before the end of the experimental incubation. The experiments were then terminated and [^{3}H]-thymidine incorporation assessed, as a measurement of cell growth. Each of the analogues was screened at 3 different

concentrations. There were 6 wells in each group and each experiment was repeated 3 or 4 times. All treatments were compared to a vehicle control; data were analyzed using analysis of variance with post-hoc Dunnett's tests for significant main effect. A 5% significance level was used throughout.

4.2.2. Bone growth activity assays (differentiation)

Osteoblasts were isolated from 20 day old fetal rat calvariae, as described above. Primary rat osteoblast cells were plated in 6-well tissue culture dishes, at a density of 5×10^4 cells/well, in Minimum Essential Medium Alpha Medium (αMEM)/10% fetal bovine serum with 5 μg/mL L-ascorbic acid-2-phosphate. When cells were confluent (approximately 3 days after plating), media were changed to αMEM/15% fetal bovine serum supplemented with 50 μg/mL L-ascorbic acid-2-phosphate and 10 mM β-glycerophosphate, and test substances were added. These supplemented media were changed twice weekly and test substances were replaced. After 21 days, the cells were fixed in 10% neutral buffered formalin, rinsed with distiled water, and the cultures stained using Von Kossa stain. Quantification of mineralized areas was performed using Bioquant image analysis software (Bioquant Image Analysis Corporation, Nashville, TN).

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Supplementary data

Supplementary data (Experimental data for the synthesis of peptides **1**–**29**, RP-HPLC and ESI-MS traces) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.05.026.

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