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Beta amino acid-modified and fluorescently labelled kisspeptin analogues with potent KISS1R activity

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Kisspeptin analogues with improved metabolic stability may represent important ligands in the study of the kisspeptin/KISS1R system and have therapeutic potential. In this paper we assess the activity of known and novel kisspeptin analogues utilising a dual luciferase reporter assay in KISS1R-transfected HEK293T cells. In general terms the results reflect the outcomes of other assay formats and a number of potent agonists were identified among the analogues, including β^2-hTyr-modified and fluorescently labelled forms. We also showed, by assaying kisspeptin in the presence of protease inhibitors, that proteolysis of kisspeptin activity within the reporter assay itself may diminish the agonist outputs.

Keywords: kisspeptin; KISS1R agonist; beta amino acids; fluorescent peptides

Introduction

Kisspeptin (*aka* metastin) and its cognate receptor, KISS1R (GPR54), have been identified in various vertebrate species [1–3] including humans [4]. Kisspeptin was found to present as several forms (KP-10, 13, 15, and KP-54) derived from a precursor peptide, which share a functionally important *N*-terminal core region. Kisspeptin's activity [5] is dictated through the KISS1 receptor (GPR54) and is a pivotal element in the neuroendocrine network governing gonadotropin secretion and is thus essential for the physiological functions of gonadotropin–releasing hormone (GnRH). Thus KISS1R agonists may be useful for the treatment of infertility, hypogonadism, and delayed puberty, and functional antagonists would be useful for the treatment of hormone – dependent cancers (prostate, breast, endometrial), ovarian hyperstimulation, contraception and precocious puberty.[6–8] However, despite recent publications, much remains unknown about the physiological role(s) and mechanisms of actions of the 'kisspeptin family of peptides' and associated receptors in veterinary and aquaculture settings. The dearth of information about how kisspeptin interacts with GPR54 is due to, in-part, limited access to appropriate kisspeptin 'mimics' for use as biological probes and suitable biological assays.

Like most proteins and peptides, the utility of kisspeptin and its peptide analogues either clinically or as pharmacological tools will be compromised by limiting physicochemical properties. Typically this means poor oral bio-availability and its short biological half-life, thus mimics or antagonists necessarily need to avoid these shortcomings. Approaches to novel GPR54 ligands (agonists and antagonists) are still in their infancy but include the following: (i) HTS for small molecules GPR54 agonists and antagonists [9–11] and (ii) Peptide analogues designed with unnatural amino acid substitutions (Table 1).[12] Among these, a series of agonist compounds have been described including d-Tyr^1-KP10,[13] other analogues by workers at Takeda (TAK series)[14] and *N*-terminally modified pentapeptides (FTM series) from Tomita et al.[12] Just one peptide antagonist has been reported known as Peptide 234.[15] Most recently, a triazol-linked family of analogues has been described including peptide 'Beltramo **3**' (Table 1).[16]

While giving promise to the idea of the development of therapeutic agents targeting GPR54, these few reports leave us with little information about the interaction between kisspeptin and GPR54. Structural information of kisspeptins is limited to conformational studies of kisspeptin-13 that suggests a helical conformation of the peptide in solution, [9] while in contrast certain modifications are consistent with favouring turn conformers. [14] In the long term, the identification of GPR54-dependent biological functions will be well served by developing a better understanding of the bioactive conformation and how it interacts with the receptor. This has been made more significant with the apparent affinity of kisspeptin peptides for the more recently identified neuropeptide FF receptors.[17] Peptidomimetic kisspeptin analogues (agonists and antagonists) are essential tools to improve our knowledge in this area.

The *in vitro* study of GPR54 agonsim has been achieved via a number of different assay formats. The majority of studies have employed calcium flux in transfected-CHO cells as measured in the FLIPR assay protocols. [12] Other assays have examined ERK1/2 phosphorylation. [13] Reporter genes have also been employed. First, Niida reported a LacZ-based system in yeast, [18] while Lee et al. utilised a c-fos-Luciferase system. [1] Kuohung

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Table 1. Reported KISS1R ligands

Name	Sequence	Reference
Kisspeptin-10	H-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2	
[dY]1-KP10	H-d-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2	[13]
KISS1-305	d-Tyr-d-Pya(4)-Asn-Ser-Phe-azaGly-Leu-Arg(Me)-Phe-NH_2[a]	[14]
TAK448	Ac-d-Tyr-Hyp-Asn-Thr-Phe-azaGly-Leu-Arg(Me)-Trp-NH_2	[14]
TAK663	Ac-d-Tyr-d-Trp-Asn-Thr-Phe-azaGly-Leu-Arg(Me)-Trp-NH_2	[14]
FTM-080	FBz-Phe-Gly-Leu-Arg-Phe-NH_2	[12]
FTM-145	FBz-Phe-Gly = Leu-Arg-Phe-NH_2[b]	[12]
Beltramo 3	Ac-Tyr-Asn-Trp-Asn-Ser-Phe-Glyψ[Tz]Leu-Arg-Phe-NH_2[c]	[16]
Peptide 234	Ac-d-Ala-Asn-Trp-Asn-Gly-Phe-Gly-d-Trp-Arg-Phe-NH_2	[15]

[a]d-Pya(4) refers to d-4-pyridinylalanine

[b]Gly = Leu refers to an alkenyl replacement for the conventional carboxamide;

[c]Glyψ[Tz]Leu refers to a triazolyl replacement for the conventional carboxamide. See references for details.

et al. described a number of assays developed for high throughput screening purposes including the use of an IP3 sensitive IP-One HTRF™ assay. [11] It is worth noting that each of these assays has a marked difference in the kinetics of the outputs and so the degree of exposure of the cells to test compounds also differs.

Here we assessed kisspeptin analogues via an alternate *in vitro* reporter assay. We transiently transfected HEK293T cell line with a human GPR54 construct (hGPR54) and monitored the activity of GPR54 using the dual luciferase reporter assay, in which the expression of firefly luciferase gene is activated by the induction of the serum response element (SRE)–mitogen–activated protein (MAP) kinase signal transduction pathway.

We have used this assay to evaluate the activity of a range of synthetic analogues of KP10. These include analogues possessing a range of novel structural motifs: peptides incorporating unusual amino acids, fluorescent KP10 analogues and cyclic peptides. [19] Kisspeptins have been shown to be degraded by serum proteases via the cleavage of the terminal Tyr-Asn and Arg-Phe bonds but also endpeptidase cleavage after Trp^{47} Phe^{50} and Gly^{51}.[20,21] As such a particular priority of this approach was to target modifications that protect the products from proteolytic degradation. We decide to examine the β^2-homoamino acid class, which might offer reduced peptidase susceptibility. Introduction of β-amino acid homologues of DNA-encoded α-amino acids has resulted in an array of interesting pharmacological and structural outcomes in peptide science, although β^2-homoamino acids have been less well studied than their β^3-homoamino acid counterparts. [22–24] We also compiled a range of fluorescent ligands, targeting a variety of fluorophores, linking chemistries and positional substitution. [25] Such ligands as well as providing useful tools for studying KISS1R pharmacology contribute to the SAR understanding of kisspeptin-related peptides.

Materials and Methods

Chemistry

N^{α}-Fmoc-protected amino acids were purchased from Auspep and ChemImpex. Rink amide resin and *O*-(1*H*-6-chlorobenzotriazol-1-y1)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HCTU) were purchased from ChemImpex. 6-Chloro-benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyClock) was purchased from Merck. Methylbenzhydrylamine (MBHA) resin, piperidine and trifluoroacetic acid (TFA) were purchased from Auspep. *N,N*-diisopropylethylamine (DIPEA), DMF, DCM, were purchased from Merck. Triisopropylsilane (TIPS) was purchased from Sigma-Aldrich. 4-Fluorobenzoic acid was purchased from Alfa Aesar. Rhodamine B isothiocyanate was obtained from Sigma-Aldrich. The azidopentanoylpiperazine-rhodamine B derivative was prepared in-house. [26,27] Cy5.5 carboxylic acid was obtained from Lumiprobe (Cat# 27090) (Florida). All chemicals were used without further purification.

Liquid Chromatography Mass Spectra (LCMS) were acquired on a Shimadzu 2020 LCMS system incorporating a photodiode array detector coupled directly into an electrospray ionisation source and a single quadrupole mass analyser. RP-HPLC was carried out at room temperature employing a Phenomenex Luna C8 (100 × 2.0 mm I.D.) column eluting with a gradient of 0–100% ACN in 0.05% aqueous TFA over 15 min at a flow rate of 0.2 ml/min. Mass spectra were obtained in positive mode with a scan range of 200–2000 m/z. Semi-preparative reverse-phase HPLC was performed using a Waters Associates liquid chromatography system (Model 600 controller and Waters 486 Tuneable Absorbance Detector) using a gradient of 0 80% ACN in 0.1% TFA over 20 min or 30 min at a flow rate of 10 ml/min on a Phenomenex Luna C8 100 Å, 10 μm (250 × 21.2 mm I.D.) or Phenomenex Luna C8 100 Å, 10 μm (100 × 21.2 mm I.D.) column.

Except where otherwise stated peptide syntheses were performed on Rink amide resin (0.3–0.7 meq/g, 100–200 mesh, 0.1 mmol scale) using conventional Fmoc-based solid phase peptide synthesis. Fmoc-protected amino acids in threefold molar excess were coupled using DMF as solvent; sixfold molar excess of diisopropylethylamine in DMF (70 ml/l) with threefold molar excess of HCTU as the activating agent for 50 min. Fmoc deprotection was carried out by treatment with 20% piperidine in DMF for 10 min. Peptide cleavage off resin was performed using a cocktail containing TFA/TIPS/DMB (92.5%:2.5%:5%) for 2 h [28]. The cleavage mixture was filtered, concentrated by a stream of nitrogen, precipitated by cold diethyl ether and centrifuged. The resulting crude product was dissolved in water/acetonitrile (1 : 1) and lyophilised overnight.

β^2-Homoamino Acid Containing Peptides

Boc-β^2-homoamino acid synthesis

General procedure for the Knoevenagel condensation

To a solution of methyl cyanoacetate **I** (1.3 eq.) and piperidine (seven drops) in MeOH (50 ml) was added the aldehyde (1 eq.)

and the reaction mixture refluxed for 16 h. The reaction was cooled to room temperature and the product precipitated out by addition of H_2O (50 ml), filtered and washed (H_2O, 10 ml) to give the product. Purification where necessary by column chromatography.

*Methyl 2-cyano-3-phenylacrylate (**IIa**) [29,30].* From benzaldehyde (2.20 ml, 24.9 mmol). White solid (3.56 g, 96%). R_f 0.66 (25% EtOAc in hexane). ^{1}H-NMR: ($CDCl_3$, 300 MHz) δ 8.26 (1H, s, CH); 7.99 (2H, d, J = 9.0 Hz, Ar. H); 7.56–7.47 (3H, m, Ar. H); 3.93 (3H, s, COOMe). Mp: 85–87 °C, lit [30] 87–89 °C.

*Methyl 2-cyano-4-methylpent-2-enoate (**IIb**).* From isobutyraldehyde (1.50 ml, 16.4 mmol) Clear oil (0.905 g, 36%). R_f 0.84 ($CHCl_3$). ^{1}H-NMR: ($CDCl_3$, 400 MHz) δ 7.43 (1H, d, J = 10.6 Hz, CH); 3.82 (3H, s, COOMe); 2.99–2.90 (1H, m, CH); 1.11 (6H, d, J = 6.6 Hz, 2 × CH_3).

*Methyl 3-(4-hydroxyphenyl)-2-isocyanoacrylate (**IIc**) [31].* From *p*-hydroxybenzaldehyde (2.40 g, 19.7 mmol). White solid (3.25 g, 81%). ^{1}H-NMR: ($CDCl_3$, 300 MHz) δ 8.21 (1H, s, CH); 7.96 (2H, d, J = 8.7 Hz, Ar. H); 6.91 (2H, d, J = 9.0 Hz, Ar. H); 3.87 (3H, s, COOMe). Mp: 213–214 °C, lit [31] 208–210 °C.

*Methyl 3-(1H-indol-3-yl)-2-isocyanoacrylate (**IId**) [32,33].* From 3-indolecarboxaldehye (3.60 g, 24.9 mmol). White solid (3.35 g, 75%). R_f 0.61 (50% EtOAc in hexane). ^{1}H-NMR: ($CDCl_3$, 300 MHz) δ 9.15 (1H, br s. NH); 8.64 (2H, m, Ar. H and CH); 7.87–7.84 (1H, m, Ar. H); 7.50–7.48 (1H, m, Ar. H); 7.34 (2H, m, Ar. H); 3.93 (1H, s, COOMe). ESI–MS (+): m/z = 227.1 $[M+H]^+$, ESI-MS (−) 225.1 $[M-H]^-$. Mp: 186.5–188.5 °C, lit [34] 165.9 °C.

General procedure for one-pot reduction and Boc protection

To a solution of the alkene (1 eq.) in MeOH (300 ml) in a 500 ml round bottomed flask fitted with a drying tube, was added $CoCl_2 \cdot 6H_2O$ (0.5 eq.) and Boc_2O (3 eq.). The mixture was cooled to 0 °C and $NaBH_4$ (14 eq.) added slowly over 2 h. The reaction was warmed to room temperature and stirred for 16–24 h. To the reaction mixture was added diethylenetriamine (1 eq.) and stirred for 30 min. The solvent was evaporated *in vacuo* and the purple residue taken up in EtOAc. The organic layer was washed with saturated aq. $NaHCO_3$, and dried over Na_2SO_4. The solvent was removed *in vacuo* and the residue purified by flash chromatography using 0–25% EtOAc in hexane to give product.

*Boc-(±)-β^2-homophenylalanine-methyl ester (**IIIa**) [35].* From **IIa** (0.500 g, 0.27 mmol). Pale yellow oil (0.185 g, 26%). R_f 0.74 (25% EtOAc in hexane). ^{1}H-NMR: ($CDCl_3$, 300 MHz) δ 7.28–7.23 (5H, m, Ar. H); 4.94 (1H, s, NH); 3.62 (3H, s, COOMe); 3.37–3.20 (2H, m, CH_2 and CH); 2.97–2.75 (3H, m, CH_2 and CH); 1.41 (9H, s, Boc).

*Boc-(±)-β^2-homoleucine-methyl ester (**IIIb**).* From **IIb** (0.679 g, 4.43 mmol). Pale yellow oil (0.321 g, 28%). ^{1}H-NMR: ($CDCl_3$, 300 MHz) δ 4.89 (1H, br. s, NH), 3.66 (3H, s, COOMe), 3.29 (1H, dd, J = 11.8, 5.6 Hz, CH_2), 3.25–2.59 (3H, m, CH_2 and CH), 1.39 (1H, s, 0.5 × CH_2), 1.29–1.20 (6H, m, 2 × CH_3); 0.87 (9H, d, J = 6.1 Hz, Boc). ESI-MS (+): m/z = 260.4 $[M+H]^+$, 282.4 $(M+Na)^+$.

*Boc-(±)-β^2-homotyrosine-methyl ester (**IIIc**).* From **IIc** (1.72 g, 8.48 mmol). Pale yellow oil (0.548 g, 16%). R_f 0.54 (50% EtOAc in hexane). ^{1}H-NMR: ($CDCl_3$, 600 MHz) δ 6.99 (2H, d, J = 8.1 Hz, Ar. H); 6.71 (2H, d, J = 7.5 Hz, Ar. H); 3.66 (3H, s, COOMe); 3.35 (1H, m, 0.5 × CH_2); 3.23 (1H, m, CH); 2.90–2.88 (2H, m, 0.5 × CH_2 and 0.5 × CH_2); 2.72 (1H, dd, J = 9.9, 15.6 Hz, 0.5 × CH_2); 1.43 (9H, s, Boc).

Di-Boc-(±)-β^2-homotyrosine-methyl ester was also obtained pale yellow oil (0.319 g, 9%). R_f 0.79 (50% EtOAc in hexane). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ 7.15 (2H, d, J = 12.0 Hz, Ar. H); 7.06 (2H, d, J = 6.0 Hz, Ar. H); 3.62 (3H, s, COOMe); 3.37–3.35 (1H, m, 0.5 × CH_2); 3.28–3.26 (1H, m, CH); 2.94–2.89 (2H, m, 0.5 × CH_2 and 0.5 × CH_2); 2.80–2.78 (1H, m, 0.5 × CH_2); 1.54 (9H, s, Boc); 1.42 (9H, s, Boc)

*Boc-(±)-β^2-homotryptophan-methyl ester (**IIId**).* From **IId** (1.00 g, 4.40 mmol). Brown oil (0.898 g, 61%). R_f 0.22 (25% EtOAc in hexane). ^{1}H-NMR: ($CDCl_3$, 300 MHz) δ 7.85 (1H, d, J = 9.0 Hz, Ar. H); 7.34 (1H, d, J = 9.0 Hz, Ar. H); 7.18 (1H, t, J = 9.0 Hz, Ar. H); 7.11 (1H, t, J = 9.0 Hz, Ar. H); 7.01 (1H, s, Ar. H); 3.67 (3H, s, COOMe); 3.45–3.29 (2H, m, CH_2); 3.20–3.10 (1H, m, CH); 3.04–2.92 (2H, m, CH_2); 1.43 (9H, s, Boc). ESI-MS (+): m/z = 333.5 $[M+H]^+$, 665.8 $[M+2H]^{2+}$.

General procedure for the ester hydrolysis

To a solution of the Boc protected methyl-ester (1 eq.) in THF (5 ml) was added a solution of lithium hydroxide (1.2 eq.) in 5 ml of H_2O. The reaction mixture was stirred at room temperature for 16 h or heated with stirring under microwave irradiation (100 °C, Power = 105 W) for 40 min. The organic solvent was removed *in vacuo,* the residue acidified with 1 M HCl to pH 2 and extracted with ethyl acetate (3 × 10 ml). The combined organic layers were washed with brine (10 ml), dried over Na_2SO_4 and the solvent removed *in vacuo* to give the product.

*Boc-(±)-β^2-homophenylalanine (**IVa**)[36–39].* From **IIIa** (0.636 mg, 2.17 mmol) Yellow solid (0.623 g, > 99%). ^{1}H-NMR: (CD_3OD, 300 MHz) δ 7.34–7.20 (5H, m, Ar. H); 3.46–3.20 (2H, m, CH_2); 3.14–2.71 (3H, m, CH and CH_2); 1.46 (9H, s, Boc). ESI-MS (−): m/z = 278.5 $[M-H]^-$, 557.7 $(2M-H)^-$. Mp: 94−96 °C.

*Boc-(±)-β^2-homoleucine (**IVb**)[37].* From **IIIb** (0.321 mg, 1.24 mmol) Pale yellow oil (0.301 g, 99%). ^{1}H-NMR: (CD_3OD, 300 MHz) δ 3.47–3.13 (2H, m, CH_2); 2.77–2.61 (1H, m, CH); 1.91–1.69 (2H, m, CH_2); 1.47 (9H, s, Boc); 0.94 (6H, d, J = 6.48 Hz, 2 × CH_3). ESI-MS (−): m/z = 244.2 $[M-H]^-$.

*Boc-(±)-β^2-homotyrosine (**IVc**).* From **IIIc** (319 mg, 1.03 mmol). Yellow oil (0.186 g, 61%). ^{1}H-NMR: (CD_3OD, 600 MHz) δ 7.00 (2H, d, J = 6.8 Hz, Ar. H); 6.71 (2H, d, J = 6.9 Hz, Ar. H); 3.43–3.33 (1H, m, 0.5 × CH_2); 3.24–3.22 (1H, m, 0.5 × CH_2); 3.13–3.08 (1H, m, CH); 2.77–2.75 (1H, m, 0.5 × CH_2); 2.66–2.63 (1H, m, 0.5 × CH_2); 1.43 (9H, s, Boc). ESI-MS (+): 318.3 $(M+Na)^+$, ESI-MS (−): m/z = 294.9 $[M=H]^-$.

*Boc-(±)-β^2-homotryptophan (**IVd**) [40].* From **IIId** (0.856 mg, 2.57 mmol). Yellow oil (0.679 g, 83%). ^{1}H-NMR: ($CDCl_3$, 300 MHz) δ 7.55 (1H, d, J = 7.7 Hz, Ar. H); 7.08 (3H, dt, J = 7.0, 14.6 Hz, Ar. H); 6.95 (1H, s, Ar. H); 3.46–3.22 (2H, m, CH_2); 3.14 (1H, dd, J = 4.9, 13.2 Hz, CH); 3.03–2.82 (2H, m, CH_2); 1.41 (9H, s, Boc). ESI–MS (−): m/z = 317.2 $[M-H]^-$.

β^2-Homoamino Acid Containing Peptide Synthesis

β^2-Homoamino acid containing peptides were prepared on MBHA resin by a mixed Boc/Fmoc-based synthesis strategy, such that after coupling with Boc-protected β^2-homoaminoacids, deprotection was performed with 100% trifluoroacetic acid. The TFA-stable Fmoc-Arg(Mtr) was used for introduction of arginine. Peptides were cleaved from the resin using a mixture of trifluoromethane sulfonic acid and TFA as previously described.[19]

Peptide Labelling

Rhodamine linked amide **12** was prepared by treatment of kisspeptin with a rhodamine B derivative activated with PyClocK and NMM in DMF for three hours. The solvent was removed *in vacuo,* the residue dissolved in a minimum volume of TFA,

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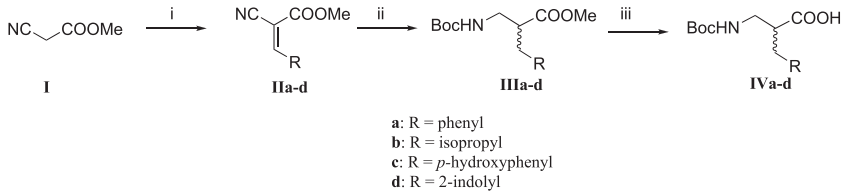

Scheme 1. *Reagents and conditions*; i, R-CHO, piperidine, MeOH, reflux 16 h; ii, $CoCl_2.6H_2O$, Boc_2O, $NaBH_4$, 24 h; iii, LiOH, THF/H_2O, RT, 16 h or mw, 100 nM, 40 min.

precipitated with ether and centrifuged to yield the crude product which was purified by RP-HPLC. RhB-KP10 **12** RT 14.94 min

Cy5.5 linked amide **13** was prepared by treatment of kisspeptin with Cy5.5 carboxylic acid activated with PyClocK and NMM in DMF for three hours. The solvent was removed *in vacuo,* the residue dissolved in a minimum volume of TFA, precipitated with ether and centrifuged to yield the crude product which was purified by RP-HPLC. Cy5.5-KP10 **13** RT 12.8 min

Rhodamine thioureas **14** and **16** were prepared by treating a solution of the peptide (0.015 mmol) in methanol with rhodamine B isothiocyanate (8 mg, 0.15 mmol) and 0.1 M Na_2CO_3 was added until a pH of 9 was attained. The reaction was stirred overnight at RT, then diluted with water (40 ml) and freeze dried. The residue was purified by RP-HPLC.

14 was obtained as two regioisomers RT 14.7, 15.1 min.

16 was obtained as two regioisomers RT 14.5, 14.9 min.

The triazolo-linked rhodamine B peptide **15** was prepared by involved dissolving the corresponding peptide-alkyne (1 eq.) in H_2O and adding a solution of the azido substituted rhodamine B derivative (4 eq.) in DMF to give a 1 : 3 ratio of H_2O to DMF. Copper sulfate (10 eq.), TBTA (10 eq.) and sodium ascorbate (10 eq.) were then added and the reaction mixed for 3 h. [27] Peptides were purified by reverse-phase preparative HPLC.

Dual Luciferase Reporter Gene Assay

Full CDS region of human GPR54 mRNA (GenBank accession number NM_032551) was amplified from FirstChoice PCR-Ready Human Brain cDNA (Ambion, Austin, TX) and cloned into a pcDNA3.1(+) expression vector (Invitrogen, Carlsbad, CA) to prepare the GPR54 expression construct (pcGPR54). HEK293-T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Alckland, NZ) supplemented with 10% fetal bovine serum (FBS), 0.1 x penicillin–streptomycin solution (iDNA, Kuala Lumpur, Malaysia) under 5% CO_2. One day before transfection, cells were plated in 24-well plates in the media without penicillin–streptomycin. Cotransfection of pcGPR54 (100 ng/well), pSRE-Luc (100 ng/well; Stratagene, La Jolla, CA), and pRL-TK vectors (25 ng/well; Promega, Madison, WI) was carried out with Lipofectamine 2000 transfection reagent (Invitrogen) overnight according to the manufacturer's instructions. The cells were serum starved in the media with 0.5% FBS for 18–20 h, and then treated with the vehicle or GPR54 analogues in the media for 6 h. The cells were harvested and the luciferase activity in the cell extracts was determined using Dual–Luciferase Reporter Assay System (Promega) in a single-tube luminometer (Sirius; Berthold Detection Systems GmbH, Pforzhein, Germany) according to the manufacturer's instruction.

Data Analysis

Luciferase induction as a percentage of maximal compound activity was calculated by setting the highest induction of each compound at 100%. Data analysis including the calculation of half effective concentration (EC_{50}) and a fit sigmoidal graph was performed using Origin 6.0 software (Microcal Software, Inc., Northampton, MA). All data are presented as mean ± SEM.

Protease Inhibitor Assay

To examine the effect of protein degradation during the assay, dual luciferase reporter gene assay was conducted with the addition of Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO). Different doses (0, 1, 2, and 5 µl) of the protease inhibitor was applied to 500 µl media and HEK293-T cells transfected with the vectors were incubated with different doses of KP-10 in the media for 6 h. The cells were harvested and the luciferase activity in the cell extracts was determined using Dual-Luciferase Reporter Assay System (Promega) in a single-tube luminometer (Sirius) according to the manufacturer's instruction.

Results

(a) Synthetic Analogues of KP-10

A variety of kisspeptin analogues were prepared as assay controls. This included a kisspeptin 10 (**1**), the d-Tyr analogue (**2**), a simplified analogue of TAK448 (**3**), an analogue of FTM-080, (**4**) and Peptide 234 (**5**). (Table 1) These peptides were synthesised using standard Fmoc solid phase peptide synthesis procedures on Rink amide resin.

In addition, we evaluated some novel peptides containing β^2-homoamino acids. The Boc-protected amino acids were prepared according to the general methods described by Pataj[41] and Caddick.[42] The first step of this synthesis (Scheme 1) was the Knoevenagel condensation between methylcyanoacetate (**I**), and the aldehydes in the presence of piperidine and methanol, to give the alkenes in 36–95% yield. One pot reduction and *N*-Boc protection of alkenes **IIa-d** were performed using cobalt-boride, which was formed *in situ* from sodium borohydride and cobalt chloride hexahydrate. Base-catalysed hydrolysis of the methyl esters **IIIa-d** under thermal or microwave heating (100 °C, 105 W) with lithium hydroxide gave the Boc-β^2-homoamino acids, Boc-β^2-hTyr, Boc-β^2-hPhe, Boc-β^2-hLeu, and Boc-β^2-hTrp (**IVa-d**) in good yields. All spectroscopic data were consistent with proposed structures and literature values, where available.

A KP10 analogue containing a β^2-homotyrosine at the *N*-terminus was prepared as well as analogues of the linear hexapeptides FTM 180 and **4**. The β-amino acids were coupled as Boc-protected derivatives. In examples that contained mid-sequence substitutions, were synthesised using a mixed Fmoc and Boc protection strategy on MBHA resin. The yields of purified peptide in these cases were quite low, in part due to poor yield of the β^2-amino acid coupling, but in addition, residual trifluoromethanesulfonic acid created problems in isolation of the

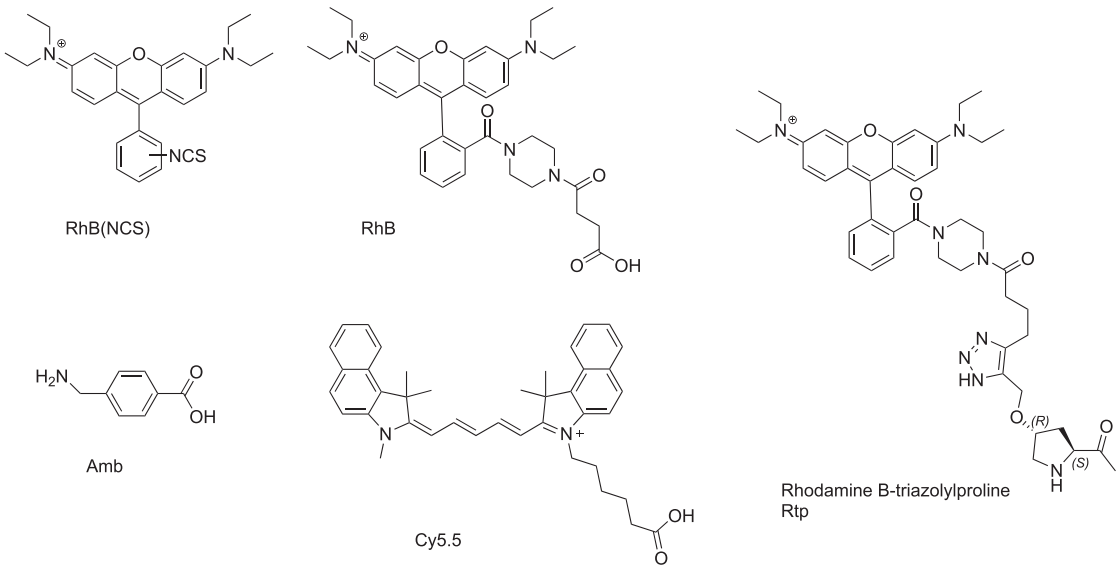

Figure 1. Chemical structure of fluorophores utilised in this study.

product from crude cleavage reaction mixtures. The products were all obtained as pairs of diastereomers, consistent with the racemic nature of the precursor β^2-homoamino acids and the mixtures could not be separated by HPLC. In the case of peptide **3**, the purification from other impurities resulted in partial resolution of the diastereomers yielding a 1 : 4 mixture of the diastereomeric pair.

We also prepared fluorescently labelled analogues where the fluorophore was appended either through the amino terminus (**12-14**) or by introduction of an appending ligand to replace Asn^4 (**15-16**). (Figure 1) While this residue is important for activity as shown by alanine replacement, we reasoned that the carboxamide side chain might be mimicked by thiourea or triazole functions, utilising linkage methods we have described in other work.[27] The fluorescent rhodamine B thiourea derivatives **14** and **16** were prepared by coupling with either rhodamine B isothiocyanate either in solution or on resin. Two regioisomeric products were obtained corresponding to the two regioisomers of the parent rhodamine isothiocyanate. The *N*-terminal rhodamine **12** and Cy5.5 carboxamides **13** were prepared by reacting with the corresponding PyClock-activated esters. The triazolyl linked rhodamine was prepared by coupling the propargyloxyproline derivative with an azido functionalised rhodamine using the copper-assisted alkyne-azide cycloaddition reaction.[27]

Biological assays

(a) *Powerful Luciferase induction by kisspeptin of KISS1R transfected HEK293T cells*

The specificity of the reporter gene assay system was confirmed by comparing hGPR54 construct (hGPR54 in a pGEM-T Easy vector) with a control plasmid. Treatment with human KP-10 (**1**) increased relative (Firefly/*Renilla*) luciferase activity in a dose–response manner (Figure 2A), while no induction was seen in the HEK293T cells without hGPR54 expression, indicating that the assay system exclusively detects the activation of exogenous GPR54 and is thus useful in the evaluation of kisspeptin analogues in the present study. The induction of luciferase activity was time dependent and shown to be maximal at 6 h of incubation. The EC_{50} of KP-10 was determined to be 13 ± 1.9 nM, in general accord with the results obtained under other assay formats.[12,13]

Reported compounds **2** and **4** were also found to be potent agonists, giving EC_{50} values in the low nanomolar range and consistent with literature reports (Peptide 2 EC_{50} 3.6 nM (receptor binding)[13]; Peptide 4 EC_{50} 3.1 nM Flipr assay[12]). Compound **3**, a simplified analogue of TAK448[14] has not been previously reported and was shown to be a particularly potent agonist, with an EC_{50} of 0.24 nM in the reporter assay. (Figure 2B–D)

It should be noted that one feature of the assays with these transiently transfected cells was that the degree of induction varied from experiment to experiment, although the EC_{50} stayed relatively constant. However, it was also noticed that the level of induction for analogues **2–3** was greater than that for kisspeptin itself in the same batch of treatment. We hypothesised that this was not due to any difference in intrinsic efficacy but due to improved stability of these compounds compared with kisspeptin in the assay conditions and this was investigated further as described later. As such the reported EC_{50} values for a peptide are referenced against the maximum induction for that same peptide. (Table 1, Figure 2)

The assay system should also be suitable to evaluate potential antagonism of GPR54, with incubation of test compounds in the presence of established agonists such as KP-10. The reported antagonist 'Peptide 234' (Ac-d-Ala-Asn-Trp-Asn-Gly-Phe-Gly-d-Trp-Arg-Phe-NH_2)[15] was tested in the assay but showed no antagonism of the KP-10 response. This was both with in-house synthesised samples and the samples obtained from the laboratory that described the peptide (R. Millar, The Queens Medical Research Institute, Edinburgh).[15] These samples did display phenotypic behaviours indicative of reported GPR54 antagonism in mouse models (data not shown). The reason for these conflicting results is unknown – the problem may be due to a technical aspect of the luciferase assay system or it may relate to an off-target effect of Peptide 234 that results in indirect but functional antagonism of Kisspeptin responses.

In the study of the modified kisppetin analogues it was observed that both β^2-homoamino acid and fluorescent labelling could have dramatic effects on KISS1R agonist activity.

The kisspeptin 10 analogue bearing an *N*-terminal β^2-hTyr substitution **6** was a potent KISS1R agonist with an EC_{50} of 0.41 nM. (Figure 2E) This shows the tolerance the receptor has for the *N*-

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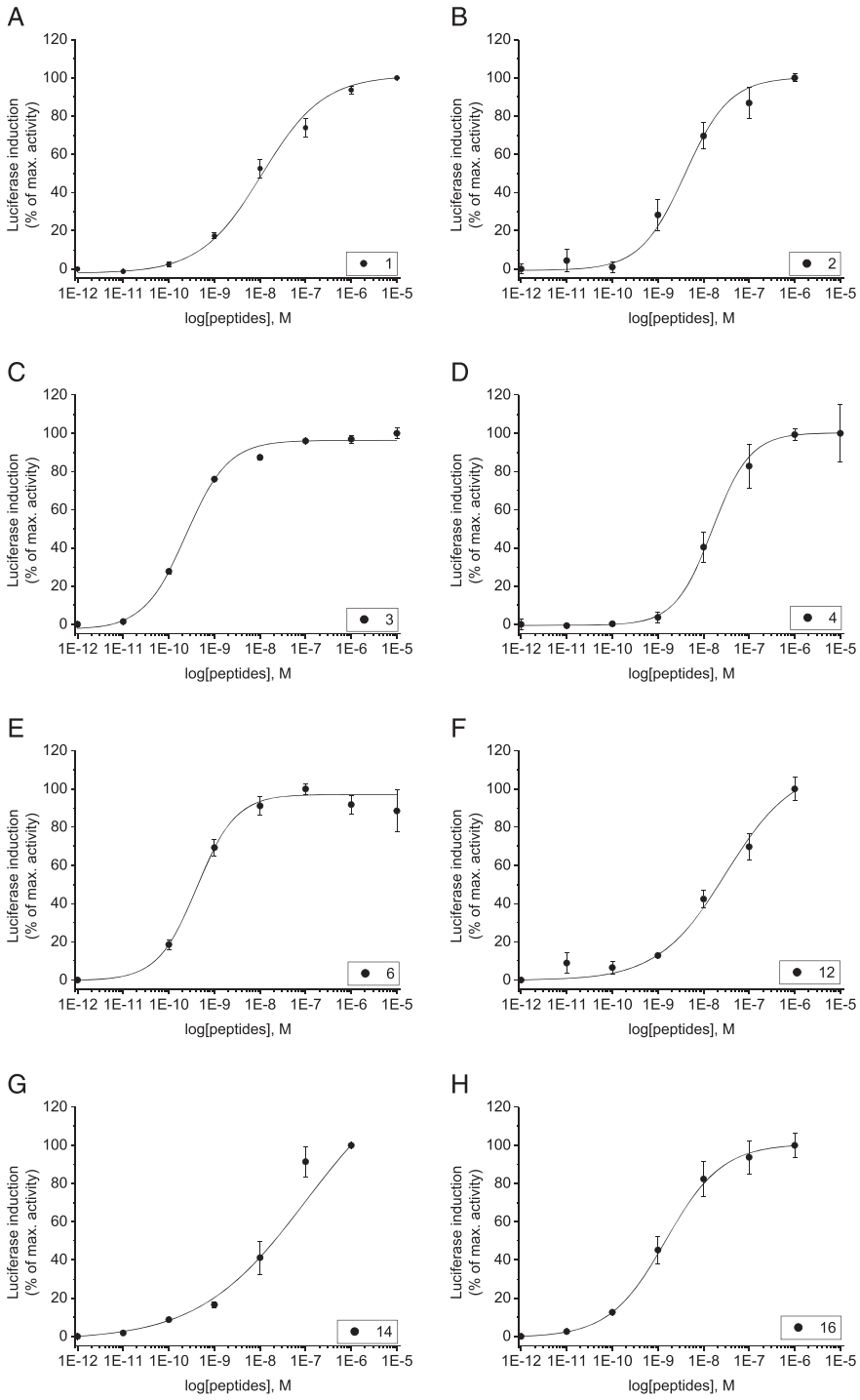

Figure 2. Dose response curves for luciferase induction by test peptides upon GPR-54 transfected HEK293-T cells. See Materials and Methods for details.

terminal modifications consistent with the potent activity of analogues **2** and **3**.

On the other hand, the β-amino acid containing analogues of FTM80 and **3** were much less active but agonist activity was seen at high concentrations of certain ligands. The compounds showed some differences in EC_{50} that must correspond to the ability to adopt the bioactive conformation found with the parent peptide. Compound **8**, where a glycine was replaced by a β-alanine (β^2-hGly), showed the strongest activity in this series with an EC_{50} of 0.6 μM. This is consistent with other studies where this region of the peptide has been amenable to certain structural changes – such as FTM145, which has a non-peptide replacement for the Phe-Gly linkage and the triazolo linked peptide Beltramo 3 (Table 1).[16,43] Note also that a series of previously published [19] cyclic peptides that adopt stable helical structures also showed no agonist efficacy in these assays (data not shown). (Figure 3)

Finally, the fluorescently labelled ligands showed a quite marked and surprising structure-activity profile. The inclusion of the rhodamine label at the *N*-terminus of KP-10, as amide **12** or thiourea **14** had little effect upon the observed potency with EC_{50} values of 30

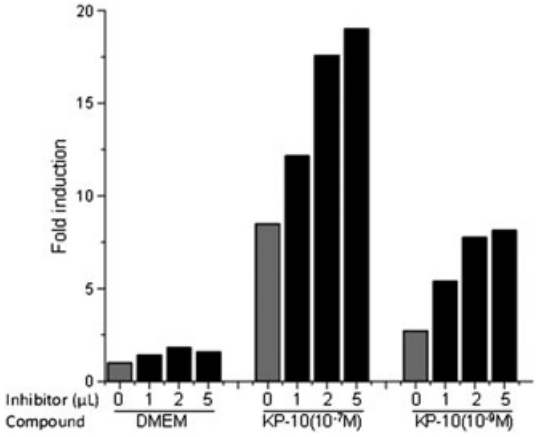

Figure 3. Comparison of luciferase induction of GPR-54 transfected HEK293-T cells by vehicle (DMEM), KP-10 (100 nM) and KP10 (1 nM) in the presence of 0, 1, 2 or 5 µl of protease inhibitor cocktail.

and 10 nM, respectively. The inclusion of the Cy5.5 label **11** completely abolished activity. This form of the Cy5.5 label has a non-sulfated benzo[e]indol-2-ylidene rendering it quite hydrophobic, which may impact on the binding affinity. (Table 2)

Introduction of a rhodamine label replacing the asparagine residue of the TAK-488 analogues resulted in a potent inhibitor **16** (EC_{50} = 1 nM) albeit less potent than the parent molecule. The introduction of a triazolyl-proline at the same position in dTyr-KP10 **15** abolished activity, a likely consequence of the conformational effect of the proline-like residue as the precursor peptide was similarly inactive (data not shown).

The variation in the extent of luciferase induction by the variety of peptide agonists described earlier was further examined. While there might be a number of mechanisms to explain 'partial' agonism displayed by KP-10, one hypothesis was that the peptide was being degraded over the course of the incubation period, such that the actual concentration of agonist was diminishing across time. As such the synthetic analogues that possessed modified amino acids might be more resistant to proteases in particular and so express agonist efficacy throughout the incubation period.

To test this hypothesis, the assay of KP-10 activity was repeated but in this case with increasing amounts of a protease inhibitor cocktail present in the culture media. The level of induction increased by over 150% at both 1 nM and 100 nM concentrations suggesting that protease activity was diminishing the full expression of agonist activity in the reporter assay.

Discussion

The development of new ligands for KISS1R is being pursued by a number of groups, as the potential for therapeutic agonists and antagonists is evident from numerous studies. In our pursuit of such ligands we have developed a functional assay format that is robust and straightforward, giving a clear dose dependent readout of agonist activity. Three reported agonists gave EC_{50} values consistent with those reported by others. The activity of peptide **2** has not been described, and showed it to be a potent agonist of GPR54. Notably, this increased potency was achieved by the introduction of D-amino acid, d-Tyr or the β-amino acid, β^2hTyr at the *N*-terminus with relatively little change to the C-terminal sequence, although it may reflect either increased binding affinity or be due to an increased stability in the time course assay. This is consistent with the reported improvement of *in vivo* potency of d-Tyr^1-KP-10.[13] We have also shown that agonist potency can be retained with the inclusion of fluorophores, also providing other potentially useful tools for studying the kisspeptin/GPR54 system. Kaneda et al. recently described alternate fluorescently labelled kisspeptin analogues through *N*-terminal derivatization of KP-14 and KP-52. The retention of agonist activity with the tetramethylrhodmaine and rhodamine green labels is consistent with our data here.[25]

We also examined the use of β-amino acids as a means of constraining the short pentapeptide agonists in the hope that it may confer metabolic stability while also retaining a bioactive conformer. The changes to the structure proved very deleterious to

Table 2. LC-MS and EC_{50} data for KISS1R peptides

Number	Sequence	ESI-MS m/z	EC_{50} /(nM)
1 (KP-10)	H-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2	1302.8	13 ± 1.9
2	H-d-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2	652.2[a]	5.3 ± 1.23
3	Ac-d-Tyr-Hyp-Asn-Thr-Phe-Gly-Leu-Arg-Trp-NH_2	1210.4	0.24 ± 0.012
4	Amb-Phe-Gly-Leu-Arg-Phe- NH_2	771.6	16 ± 5.6
5	Ac-d-Ala-Asn-Trp-Asn-Gly-Phe-Gly-d-Trp-Arg-Phe-NH_2	1295.6	—
6	H-β^2hTyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2	1300.8	0.41 ± 0.065
7	FBz-β^2hPhe-Gly-Leu-Arg-Phe- NH_2	774.6	Inactive
8	FBz-Phe-βAla-Leu-Arg-Phe- NH_2	774.7	600 ± 140
9	Amb-Phe-Gly-β^2hLeu-Arg-Phe- NH_2	786.0	>1000[b]
10	FBz-Phe-Gly-Leu-Arg-β^2hPhe- NH_2	774.3	Inactive
11	FBz-Phe-Gly-Leu-Arg-β^2hTrp- NH_2	812.9	>1000[b]
12	RhB-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2	948.9[a]	31 ± 19.2
13	Cy5.5-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2	934.7[a]	Inactive
14	RhB(NHCS)-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2	902.10[a]	10 ± 11.0
15	H-d-Tyr-Asn-Trp-Rtp-Ser-Phe-Gly-Leu-Arg-Phe-NH_2[c]	989.0[a]	Inactive
16	Ac-d-Tyr-Hyp-Dap(γSCNH-RhB)-Thr-Phe-Gly-Leu-Arg-Trp-NH_2	842.2[a]	1.0 ± 0.49

[a]ESI-MS m/z = $(M + 2H)^{2+}$;
[b]Luciferase induction still rising at maximum concentration (10 µm) tested;
[c]Rtp = rhodamine-triazolylproline (Figure 1) [27]

agonist efficacy, although in one case agonist activity was obtained at high concentrations. In principle, these changes can be compared with FTM145, a recently described peptidomimetic, where the Gly-Leu dipeptide unit was replaced by an *E*-alkene dipeptide isostere retaining the affinity of the parent peptide. However its EC_{50} of 300 nM was substantially poorer than the parent peptide.[43]

One of the interesting results identified across the course of these experiments was that the degree of agonist-induced luciferase activity was lower for kisspeptin than some other synthetic analogues irrespective of the EC_{50} of the compound. This an important feature to note as in some respects it can confound the results of single point assays in screening programmes. The luciferase activity acquired over a 6 h incubation of agonist will represent a combination of accumulated acute receptor activation events. Time-dependent loss of agonist due to degradation processes would be expected to result in lower luciferase expression even with a full agonist. The present results show that incubating the cells with a conventional protease inhibitor mixture yielded a significant rise of luciferase-related activity suggesting that proteolytic activity was responsible for the reduced induction of the native peptide. The use of these cell based assays might well be coupled to the use of competition binding assays, possibly utilising the fluorescently labelled kisspeptin analogues described here, to assist in attribution of the activity in compound screens. Equally, an *in vitro* assay that identifies protease susceptibility as part of the readout could be a useful tool in peptide optimization.

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