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A new class of pentapeptide KISS1 receptor agonists with hypothalamic–pituitary–gonadal axis activation

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ABSTRACT

The kisspeptin (Kp, Kp-54, metastin)/KISS1R system plays crucial roles in regulating the secretion of gonadotropin-releasing hormone. Continuous administration of nonapeptide Kp analogs caused plasma testosterone depletion, whereas bolus administration caused strong plasma testosterone elevation in male rats. To develop a new class of small peptide drugs, we focused on stepwise N-terminal truncation of Kp analogs and discovered potent pentapeptide analogs. Benzoyl-Phe-azaGly-Leu-Arg(Me)-Trp-NH_2 (**16**) exhibited high agonist activity for KISS1R and excellent metabolic stability in rat serum. A single injection of a 4-pyridyl analog (**19**) at the N-terminus of **16** into male Sprague Dawley rats caused a robust increase in plasma luteinizing hormone levels, but unlike continuous administration of nonapeptide Kp analogs, continuous administration of **19** maintained moderate testosterone levels in rats. These results indicated that small peptide drugs can be successfully developed for treating sex hormone deficiency.

KISS1R (GPR54, OT7T175, AXOR12) is a G protein-coupled receptor (GPCR) highly expressed in the brain, including in the hypothalamus, pituitary, and the peripheral regions.[1–3] In 2001, metastin/kisspeptin [Kp, Kp-54, metastin(1–54)], the endogenous ligand of KISS1R, was discovered as the product of the gene KISS1.[4–6] Several studies on the function of the KISS1R and Kp suggested that KISS1R plays an important role in reproduction and pubertal development by regulating the hypothalamic–pituitary–gonadal (HPG) axis.[7,8] Kp potently induced gonadotropin-releasing hormone (GnRH) secretion in mammals and non-mammalian species.[9,10] Our previous studies indicated that a single administration of Kp elevated plasma follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in male rats.[11] In contrast, chronic administration of Kp and its analogs caused a profound decrease in plasma testosterone and LH levels below the lowest limit of detection.[12–15] These findings suggested the possibility of using KISS1R agonists for the treatment of sex hormone-dependent disorders, including hypogonadism, prostate cancer, and endometriosis, by activating or inhibiting the HPG axis.

Native Kp contains 54 amino acid residues, but a C-terminal decapeptide, kisspeptin-10 [Kp-10, metastin (45–54), Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2], has 3–10 times greater activity in vitro.[4] Although Kp-10 has potent activity, its susceptibility to enzyme cleavage limits its utility. Several groups including ours have reported that rational modification of Kp-10 increased its metabolic stability.[13–23] Amino acid substitution converted Kp-10 into a nonapeptide analog, D-Tyr-D-Pya(4)-Asn-Ser-Phe-azaGly-Leu-Arg(Me)-Phe-NH_2 (**1**), that exhibited testosterone-suppressive activity as potent as Kp.[13,22,23] Both substitutions of D-Tyr at position 45 and N^{ω}-methylarginine [Arg(Me)] at position 53 of Kp-10 were acceptable for KISS1R recognition while avoiding N- and C-terminal degradation, respectively.[22,23] Replacement of Gly at position 51 with an azaglycine (azaGly) residue improved metabolic stability for the 50–51 and 51–52 bonds, which was expected to provide resistance to serum proteases.[23] Metabolic stability was subsequently improved by deletion of Asn at position 46 and substitution of Trp at position 47.[13] Further optimization studies revealed a novel class of anti-prostate cancer drug candidates, TAK-683 (Ac-D-Tyr-D-Trp-Asn-Thr-Phe-azaGly-Leu-Arg(Me)-Trp-NH_2) and TAK-448 (MVT-602, Ac-D-Tyr-Hyp-Asn-Thr-Phe-azaGly-Leu-Arg(Me)-Trp-NH_2).[14,15,24,25] In this paper, we describe a synthetic study to evaluate reduced-length analogs of Kp-10, which may allow us to transform these peptides into a new class of small molecules with different in vivo profiles in terms of the effect on activation/suppression of the HPG axis.

First, to determine whether shorter analogs can be created, we focused on the modification of the N-terminal region of Trp[47] analog (**2**)

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of **1**. All peptides were synthesized via standard 9-fluorenylmethoxycarbonyl (Fmoc)-based solid phase peptide synthesis using on-resin construction of azaGly and Arg(Me) residues at positions 51 and 53, respectively, according to a previous report.[13] N-terminl acylation of peptides was performed by coupling of carboxylic acid activated by *N,N′*-diisopropylcarbodiimide (DIPCDI)/1-hydroxy-7-azabenzotriazole (HOAt) or by acetylation with acetic anhydride. N-terminal benzyl peptide was prepared via on-resin reductive amination using benzaldehyde and sodium cyanoborohydride. After final cleavage with trifluoroacetic acid (TFA)-*m*-cresol-thioanisole-H_2O-1,2-ethanedithiol-triisopropylsilane (80:5:5:5:2.5:2.5), purification was accomplished using reversed-phase high-performance liquid chromatography (RP-HPLC), which separated peptides with a linear gradient increase of acetonitrile concentration in 0.1% (v/v) TFA. The purity of each peptide was confirmed by analytical RP-HPLC and characterized using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). All final compounds were purified to homogeneity of ≥95% as indicated by RP-HPLC analysis with UV detection at 210 and 254 nm and the absence of co-eluting impurities (heterogeneous peaks) was confirmed via LC-MS analysis. The N-terminal benzyl peptide was obtained as a mono-benzylated peptide after HPLC separation of mono-benzylated and di-benzylated peptides. The agonist activity [i.e., the ligand-induced elevation of Ca^{2+} in Chinese hamster ovary (CHO) cells expressing human KISS1R] of all peptides was determined using a fluorometric imaging plate reader (FLIPR) and the results were expressed as EC_{50} values (nM) (Table 1).

The data indicated that deletion of the N-terminal D-Tyr^{46} residue of **2** caused a considerable decrease in agonist activity (**3**), but acetylation of the N-terminal amino group in **4** slightly restored this activity (Table 1, Fig. 1). Moreover, further recovery of the agonist activity was observed in **5** after deletion of the amino group of **3**. Other N-terminal modifications of heptapeptide analogs, such as replacement of the indole group with a phenyl (**6**) or 4-pyridyl (**7**) group, resulted in retention of agonist activity. Various substitutions (for example, introduction of a dipeptide, amino acid, or hydrophobic/hydrophilic acyl group) were found to be permissible for the N-terminal group of **5** (data not shown), which suggested that the N-terminal group was not well recognized by KISS1R. We then deleted the amino acids at positions 48 and 49 to determine the importance of the resulting short-length analogs.

The single amino acid deletion at position 48 (**8**) caused a drastic decrease in agonist activity compared to **5**. However, the peptide resulting from further amino acid deletion at position 49 (**9**), which corresponded to an N-terminal indol-3-ylpropanoyl analog of the C-terminal pentapeptide of **2**, exhibited much higher agonist activity than **8**. Subsequent deletion of the N-terminal 3-(indol-3-yl)propanoyl group of **9** dramatically decreased its activity (**10**), suggesting that the N-terminal functional group of the pentapeptide analog had a critical role in KISS1R activation. Detailed structure-activity relationship (SAR) studies of the N-terminal moiety of pentapeptide analogs found that analogs with a diversity of acyl groups [i.e., acetyl (**11**), n-hexanoyl (**12**), and benzoyl (**13**)] exhibited a range of agonist activity that was comparable to that of **9**. Benzyl substitution (**14**) resulted in slightly inferior, yet acceptable, KISS1R activation.

In previous studies, substitution of Phe with Trp at position 54 of Kp-10 [metastin(45–54)] increased the binding affinity and agonist activity of the analog for the KISS1R.[14,16] Therefore, substitution with Trp^{54} in pentapeptide analogs, **9** and **13**, to yield **15** and **16** resulted in increased affinity and agonist activity. Accordingly, we performed further N-terminal modification of the pentapeptide analog with Trp substitution at position 54.

To investigate the effects of reducing peptide length on metabolic

Table 1
Structures, biological activities, and HPLC retention times of Kp analogs.

R-AA^{45}-AA^{46}-AA^{47}-AA^{48}-AA^{49}-Phe-AA^{51}-Leu-AA^{53}-AA^{54}-NH_2

Compound	R	AA^{45}	AA^{46}	AA^{47}	AA^{48}	AA^{49}	AA^{51}	AA^{53}	AA^{54}	Agonist activity[a] EC_{50} (nM)	RP-HPLC[b] t_{ret} (min)
metastin(45–54)		Tyr	Asn	Trp	Asn	Ser	Gly	Arg	Phe	0.087	–
1	H	–	D-Tyr	D-Pya(4)	Asn	Ser	azaGly	Arg(Me)	Phe	0.27 (0.074–0.99)	–
2	H	–	D-Tyr	Trp	Asn	Ser	azaGly	Arg(Me)	Phe	0.057 (0.018–0.17)	5.54
3	H	–	–	Trp	Asn	Ser	azaGly	Arg(Me)	Phe	7.7 (2.2–28)	5.03
4	Ac	–	–	Trp	Asn	Ser	azaGly	Arg(Me)	Phe	2.1 (0.96–4.6)	5.76
5	3-(indol-3-yl)propionyl	–	–	–	Asn	Ser	azaGly	Arg(Me)	Phe	0.13 (0.020–0.86)	5.95
6	3-phenylpropionyl	–	–	–	Asn	Ser	azaGly	Arg(Me)	Phe	0.17 (0.067–0.44)	5.90
7	3-(4-pyridyl)propionyl	–	–	–	Asn	Ser	azaGly	Arg(Me)	Phe	1.5 (0.48–4.8)	4.51
8	3-(indol-3-yl)propionyl	–	–	–	–	Ser	azaGly	Arg(Me)	Phe	8.2 (3.4–20)	6.17
9	3-(indol-3-yl)propionyl	–	–	–	–	–	azaGly	Arg(Me)	Phe	0.62 (0.16–2.3)	6.58
10	H	–	–	–	–	–	azaGly	Arg(Me)	Phe	130 (93–170)	4.29
11	Ac	–	–	–	–	–	azaGly	Arg(Me)	Phe	0.33 (0.19–0.57)	5.02
12	n-hexanoyl	–	–	–	–	–	azaGly	Arg(Me)	Phe	1.4 (0.44–4.3)	6.66
13	benzoyl	–	–	–	–	–	azaGly	Arg(Me)	Phe	0.60 (0.28–1.2)	6.21
14	benzyl	–	–	–	–	–	azaGly	Arg(Me)	Phe	0.89 (0.23–3.4)	5.20
15	3-(indol-3-yl)propionyl	–	–	–	–	–	azaGly	Arg(Me)	Trp	0.022 (0.0072–0.066)	6.67
16	benzoyl	–	–	–	–	–	azaGly	Arg(Me)	Trp	0.024 (0.0073–0.079)	6.29
17	2-pyridylcarbonyl	–	–	–	–	–	azaGly	Arg(Me)	Trp	0.037 (0.0099–0.14)	6.02
18	3-pyridylcarbonyl	–	–	–	–	–	azaGly	Arg(Me)	Trp	0.16 (0.037–0.67)	4.69
19	4-pyridylcarbonyl	–	–	–	–	–	azaGly	Arg(Me)	Trp	0.068 (0.023–0.20)	4.48
20	3-furanylcarbonyl	–	–	–	–	–	azaGly	Arg(Me)	Trp	0.11 (0.021–0.58)	5.88
21	2-pyrolylcarbonyl	–	–	–	–	–	azaGly	Arg(Me)	Trp	0.23 (0.18–0.31)	5.91
22	4-imidazolylcarbonyl	–	–	–	–	–	azaGly	Arg(Me)	Trp	0.24 (0.062–0.90)	4.47
23	phenylacetyl	–	–	–	–	–	azaGly	Arg(Me)	Trp	0.27 (0.048–1.5)	6.49
24	cyclohexanoyl	–	–	–	–	–	azaGly	Arg(Me)	Trp	0.12 (0.017–0.82)	6.70
25	propionyl	–	–	–	–	–	azaGly	Arg(Me)	Trp	0.13 (0.036–0.49)	5.52
26	isobutyryl	–	–	–	–	–	azaGly	Arg(Me)	Trp	0.058 (0.013–0.26)	5.88
27	cyclopropylcarbonyl	–	–	–	–	–	azaGly	Arg(Me)	Trp	0.028 (0.0081–0.096)	5.70

[a] EC_{50} values [nM (95% confidence interval)] of [Ca^{2+}] increasing activities of all peptide analogs were evaluated in CHO cells expressing human KISS1R.
[b] Retention times (t_{ret}) of peptide analogs were measured via RP-HPLC. Elution conditions: linear density gradient elution on Merck Chromolith® Performance RP-18e (4.6 × 100 mm), with eluents A/B = 95/5–35/65 (10 min), using 0.1% TFA in water as eluent A and 0.1% TFA-containing acetonitrile as eluent B; flow rate: 3.0 mL/min.

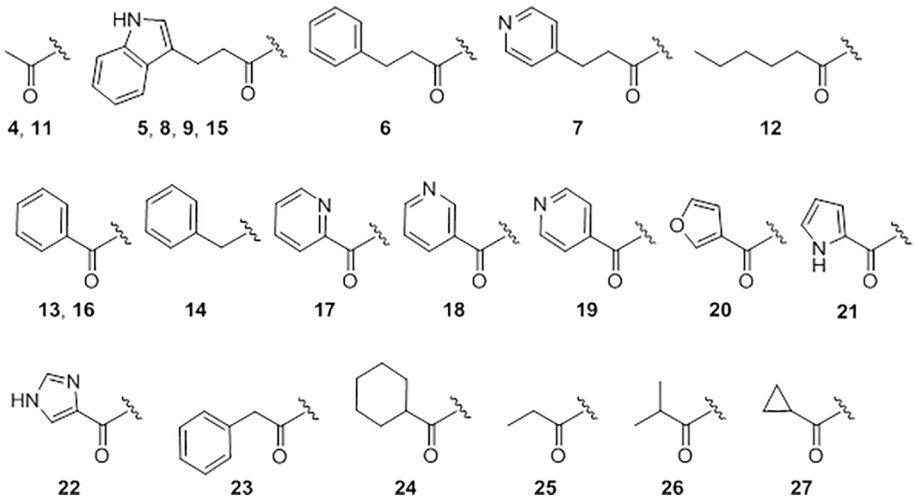

Fig. 1. Structures of the N-terminal moieties (R) of Kp analogs.

stability, the residual ratio of Kp-10 [metastin(45–54)], **1**, and **16** after incubation in Sprague-Dawley (SD) rat serum at 37 °C was analyzed via high-performance liquid chromatography-tandem mass spectrometry (LC/MS/MS). The serum stability assay was initiated with the addition of 10 µL of aqueous peptide solution (10 µg/mL) to 990 µL of pre-incubated SD rat serum. The mixed solution was incubated at 37 °C and 100-µL aliquots were sampled at 0, 5, 15, 30, 60, and 120 min. Each aliquot was mixed with 10 µL of 0.2% aqueous solution of formic acid/methanol (1:4, v/v) and 150 µL of acetonitrile. After centrifugation of the mixture at 15000 rpm for 5 min, 200 µL of supernatant was mixed with 20 µL of 0.2% aqueous solution of formic acid/methanol (1:4, v/v) containing an internal standard and 300 µL of 0.4% aqueous solution of acetic acid. Twenty microliters of supernatant from each sample was collected after centrifugation at 15000 rpm for 5 min and injected on LC/MS/MS.

The residual ratio of each peptide after incubation is shown in Table 2. Metastin(45–54) degraded quickly (with a half-life of less than 5 min) and was undetectable after 15 min. Regarding the nonapeptide analog (**1**), which was a metastin(45–54) derivative with several substitutions of amino acid to increase its metabolic stability, 70% of the initial concentration remained after 2 h of incubation. Compound **16**, the pentapeptide analog with Trp residue in addition to the same substitution as **1**, displayed excellent stability and 91% of intact peptide remained after a 2-h incubation. The efficient strategy for further improving metabolic stability was reducing the peptide length of the Kp analog containing Trp^{54} substitution. In studies on nonapeptide analogs, replacing Phe^{54} with Trp^{54} improved the mean residence time (MRT) values in rat intravenous pharmacokinetic studies.[14] The improvement of MRT appears to be attributed to the enhancement of protease resistance in the circulation.

Table 2
Residual ratio of Kp analogs after incubation in SD rat serum.

Time (min)	Residual ratio (%)[a]		
	Metastin(45–54)	**1**	**16**
0	100	100	100
5	1.5	98	98
15	< 0.5	97	97
30	< 0.5	95	99
60	< 0.5	89	95
120	< 0.5	70	91

[a] The stability index of synthetic metastin derivatives, percent of compound remaining after incubation at initial concentrations of 0.1 µg/mL, was determined at 37 °C in IDS/SD rat serum (n = 2).

Compound **16** showed good agonist activity and superior biological stability in rat serum, but had low solubility in water (< 10 mg/mL) and saline (< 0.1 mg/mL). To improve the physicochemical profile of **16**, derivatives with an N-terminal hetero aromatic, alicyclic, or aliphatic group (**17**–**27**) were synthesized and subjected to RP-HPLC analysis to estimate the hydrophobicity of peptide from their retention times.[26] After substitution with six-membered heteroaromatic ring systems, the 2-pyridylcarbonyl group (**17**) showed good agonist activity, but had a retention time similar to that of **16** on RP-HPLC, and the 3-pyridylcarbonyl group (**18**) attenuated agonist activity. Finally, **19**, with a 4-pyridylcarbonyl group, showed agonist activity as potent as **16** and had a short retention time on RP-HPLC. In fact, **19** had very good solubility in H_2O (> 20 mg/mL) and saline (> 1 mg/mL).

Among peptides with a five-membered heteroaromatic ring (**20**–**22**), only **22** had a shorter retention time, but also had a slightly decreased agonist activity. Compound **23**, which has a phenylacetyl group at the N-terminus, exhibited more than 10 times lower potency compared with **16**. Peptides with alicyclic and aliphatic moieties, cyclohexanoyl (**24**), propionyl (**25**), isobutyryl (**26**), and cyclopropanoyl (**27**), exhibited good agonist activities. In particular, the cyclopropanoyl group (**27**) expressing σ-aromaticity had a higher activity comparable with the peptide containing a benzoyl group (**16**), though these peptides were less hydrophilic. The results suggested that arylcarbonyl and α-branched structures were much more beneficial for receptor activation than hydrophobicity, aromaticity, and hydrogen bonding ability of the N-terminal moiety.

Our successful discovery of pentapeptide analogs [R-Phe-azaGly-Leu-Arg(Me)-Trp-NH_2] without N-terminal aromatic modification contrasts the previous findings of others,[17] who showed the importance of the N-terminal aromatic ring for KISS1R activation in their SAR study of pentapeptide Kp analogs (R-Phe-Gly-Leu-Arg-Trp-NH_2). This inconsistency led us to hypothesize that the azaGly and/or Arg(Me) residues affected molecular fitting to the KISS1R.

The testosterone-suppressive effect of pentapeptide analogs was evaluated in male SD rats after a 6-day sustained subcutaneous administration. Compound **16** showed low solubility in aqueous media, for the in vivo study; therefore, we selected **19** because it had water solubility suitable for a test compound. The EC_{50} value of **19** for rat KISS1R (1.2×10^{-9} M) was 7-fold higher than that of the nonapeptide, **1** (1.7×10^{-10} M). In previous studies, continuous administration of **1** and leuprolide significantly reduced plasma testosterone levels at a dose of 1 nmol/h for 13 days in male rats (plasma concentrations of **1** and leuprolide were 1.1 and 3.2 ng/mL, respectively).[13] In this study, plasma testosterone suppression by leuprolide at a higher dose of 2 nmol/h was used as a positive control (Fig. 2A). Testosterone

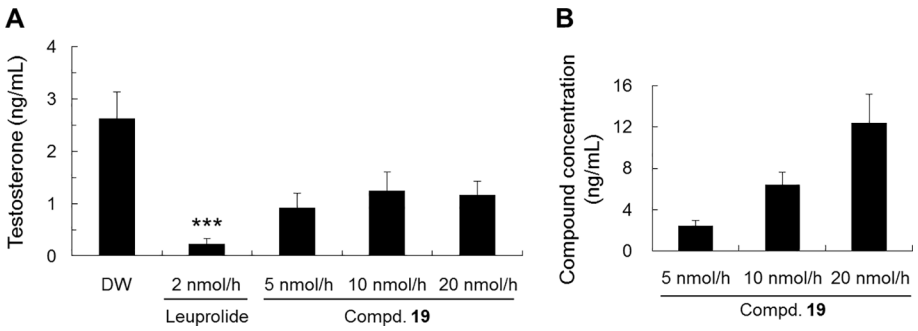

Fig. 2. (A) Plasma testosterone-suppressive activities of leuprolide (2 nmol/h) and **19** (5, 10, and 20 nmol/h) when administered continuously in male rats. ALZET osmotic pumps with 5, 10, or 20 mM aqueous solution of **19** were implanted subcutaneously in five 9-week-old Crl:CD (SD) male rats. After 6 days, the plasma testosterone level of each rat was measured using radioimmunoassay. Data are shown as means ± standard deviation. The symbol *** indicates $P < 0.0001$ vs. distilled water (DW)-treated control by Dunnett's test. (B) Steady-state plasma concentrations of **19** after a 6-day continuous administration in five male SD rats at 5, 10, and 20 nmol/h. Plasma samples were collected, purified by protein precipitation with organic solvent, and subjected to LC/MS analysis to obtain the plasma concentration of the peptides. Data are shown as means ± standard deviation.

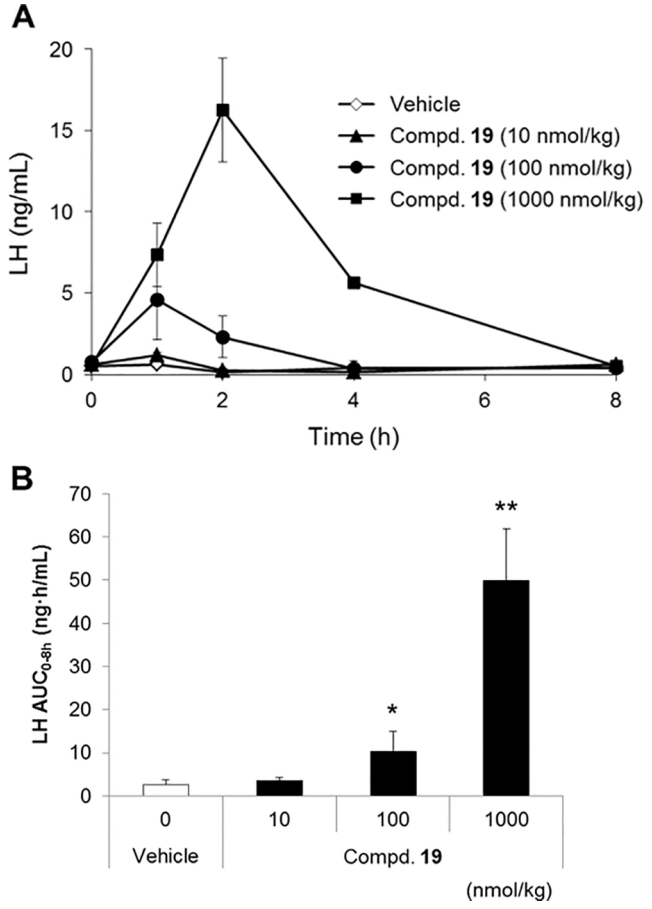

Fig. 3. LH-releasing activity of **19** after single-bolus injection in rats. Compound **19** was subcutaneously administered at 10, 100, and 1000 nmol/kg in five 9-week-old Crl:CD (SD) male rats. (A) Plasma LH levels were measured in saline-treated (vehicle) and **19**-treated rats 1, 2, 4, and 8 h after injection. Data are shown as means ± standard deviation. (B) Area under the plasma concentration-time curve of LH from time 0 to 8 h [AUC_{0-8h} (ng·h/mL)] after bolus administration of **19** for each rat was calculated. All data are means ± standard deviation ($n = 5$). $^{*}P < 0.025$, $^{**}P < 0.0005$ vs. vehicle as assessed via one-tailed Williams' test.

inhibition by **19** was only moderate at all tested doses and was incomplete even at the highest dose of 20 nmol/h, although the plasma concentration of **19** increased in a dose-dependent manner (Fig. 2B). Conversely, single-bolus administration of **19** at doses between 10 and 1000 nmol/kg elevated plasma levels of luteinizing hormone (LH) with a clear dose dependency in male SD rats (Fig. 3A). Compound **19** showed significant LH-releasing activity at doses greater than 100 nmol/kg (Fig. 3B).

With once-daily repeated dosing of nonapeptide Kp analogs (TAK-448 and TAK-683) in male rats, plasma LH and testosterone levels substantially increased on the first day, but this response was attenuated at days 4 and 7.[27] The results of chronic and repeated dosing with nonapeptide analogs suggested that continuous exposure to Kp analogs might induce a desensitization of the KISS1R. On the other hand, pentapeptide analogs, for example **19** in this study, may have the opposite effect, that is, to enhance sex hormone release without attenuation owing to repeated dosing. More suitable Kp analogs for treating sex hormone deficiency will require further investigation of the biological profiles of pentapeptide analogs.

In this study, we synthesized short-length Kp analogs with the amino acid substitutions of azaGly[51] and Arg(Me)[53] by stepwise N-terminal truncation of nonapeptide analogs (**1** and **2**). Reduction of peptide length resulted in **16**, which had improved metabolic stability in rat serum. N-terminal replacement in **16** led to **19** with KISS1R agonist activity comparable to that of Kp-10 [metastin(45–54)] and good aqueous solubility. Compound **19** elevated the plasma testosterone levels after a single subcutaneous administration in rats and had similar efficacy to **1**. The small ligand structure of **19** may allow the creation of a pharmacophore model for future development of KISS1R agonists of small-sized molecules for treating sex-hormone dependent diseases.

Abbreviations

Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature.[28,29] Amino acid symbols denote the L-configuration unless indicated otherwise. Ac, acetyl, Arg(Me), N^{ω}-methylarginine; azaGly, azaglycine; CHO, Chinese hamster ovary; DMF, *N,N*-dimethylformamide; DIPCDI, *N,N′*-diisopropylcarbodiimide; FLIPR, fluorometric imaging plate reader; Fmoc, 9-fluorenylmethoxycarbonyl; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; GPCR, G protein-coupled receptor; HOAt, 1-hydroxy-7-azabenzotriazole; HPG, hypothalamic–pituitary–gonadal; Hyp, *trans*-4-hydroxyproline; Kp, kisspeptin; LC/MS/MS, high performance liquid chromatography-tandem mass spectrometry; LH, luteinizing hormone; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Pya(4), 3-(4-pyridyl)alanine; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

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Appendix A. Supplementary data

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