Design and Synthesis of Fluorescent Probes for GPR54

Masato Kaneda[a], Ryosuke Misu[a], Hiroaki Ohno[a], Akira Hirasawa[a], Nahoko Ieda[b], Yoshihisa Uenoyama[b], Hiroko Tsukamura[b], Kei-ichiro Maeda[c], Shinya Oishi[a,*], Nobutaka Fujii[a,*]

[a] Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

[b] Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

[c] Graduate School of Agricultural and Life Sciences, the University of Tokyo, Tokyo 113-8657, Japan

*Corresponding Authors:

Shinya Oishi, Ph.D. and Nobutaka Fujii, Ph.D.

Graduate School of Pharmaceutical Sciences

Kyoto University

Sakyo-ku, Kyoto, 606-8501, Japan

Tel: +81-75-753-4551; Fax: +81-75-753-4570,

E-mail (S.O.): soishi@pharm.kyoto-u.ac.jp; E-mail (N.F.): nfujii@pharm.kyoto-u.ac.jp

Abstract

Kisspeptins are neuropeptides that induce the secretion of gonadotropin-releasing hormone via the activation of the cognate receptor, G-protein coupled receptor 54 (GPR54). The kisspeptin-GPR54 axis is associated with the onset of puberty and the maintenance of the reproductive system. In this study, several fluorescent probes have been designed and synthesized for rat GPR54 through the modification of the N-terminus of rat kisspeptins to allow for the visualization of the expression and localization of kisspeptin receptor(s) in living cells and native tissues. The tetramethylrhodamine (TMR) and rhodamine green (RG)-labeled kisspeptins exhibited good binding and agonistic activities towards GPR54, and the results of the application studies demonstrated that these fluorescent probes could be used effectively for the detection of GPR54 receptors in flow cytometry and confocal microscopy experiments.

Keywords: fluorescent probe; GPR54; kisspeptin; RFamide

1. Introduction

Kisspeptins are neuropeptides belonging to the RFamide peptide family, and are the proteolytic products of a protein encoded by the *KISS1* metastasis suppressor gene. Kisspeptins induce the secretion of gonadotropin-releasing hormone (GnRH) from GnRH neurons via the stimulation of G-protein coupled receptor 54 (GPR54), which consequently stimulates the release of luteinizing hormone (LH) from the anterior pituitary.[1-5] Kisspeptin-GPR54 signaling regulates the onset of puberty and is also responsible for the maintenance of the reproductive system.[6-8] A large number of studies have been reported in the literature concerning the expression of *Kiss1* mRNA in the hypothalamus,[2,3,9,10] whereas only a limited number of reports have been published pertaining to the expression and localization of GPR54. Irwig *et al.* reported the co-expression of GnRH mRNA and GPR54 mRNA in the cells of forebrain using double-label in situ hybridization method.[2] Messager *et al.* reported the co-localization of GPR54 and GnRH neurons in preoptic hypothalamic area of *GPR54*-null transgenic mice.[3] Despite these efforts, there have been no reports concerning the direct detection of GPR54 expression in the central nervous system (CNS) with immunohistochemistry because the specific antibody reagents are unavailable.

Full-length rat kisspeptin (kisspeptin-52, Kp-52) is a peptide composed of 52 amino acid residues, which has a Cys4-Cys18 disulfide bridge at its N-terminal region. The endogenous C-terminal peptides kisspeptin-14 (Kp-14) and kisspeptin-13 (Kp-13) exhibit a similar level of biological activity to that of full-length kisspeptin.[11,12] The minimal sequence required for GPR54 binding and activation in kisspeptins is a C-terminal peptide composed of 10 amino acid residues (Kp-10).[11] A number of potent GPR54 agonists have been reported to date based on studies directed towards developing a deeper understanding of the structure-activity relationships (SARs) of kisspeptins.[13-19] FTM080 is a 5-residue GPR54 agonist peptide.[14] The N-terminal of this peptide was modified with a 4-fluorobenzoyl group, whereas the C-terminal Phe residue in the RF amide motif was substituted with Trp. TAK-448 is a 9-residue peptide that contains multiple modifications of kisspeptin-10, and is the first investigational GPR54 agonist to have been reported in the literature.[18,19] The peripheral

administration of kisspeptin-based peptides led to an increase in the levels of circulating LH and testosterone in male rats,[5,20,21] as well as enhanced ovulation in musk shrews.[22] The chronic administration of kisspeptin-based peptides to male rats has also been reported to lead to a reduction in gonadotropin and testosterone levels.[19] Based on these reports, kisspeptins and their derivatives could be used as therapeutic agents for the treatment of reproductive disorders caused by the impaired regulation of GnRH/gonadotropin secretion.

We recently developed kisspeptin-based photoaffinity probes, where a photoreactive functional group was conjugated to potentially interactive residues in the sequence.[23] In this particular study, we used a panel of different probes to successfully identify several receptor binding residues for GPR54. Although these potent photoaffinity probes were suitable for the detection of GPR54 by Western blotting analysis, they could not be used for direct staining with live-cell imaging techniques. In the current study, we have designed and synthesized fluorescent probes based on Kp-52 and Kp-14 to allow for the expression and localization of GPR54 to be visualized in vitro using flow cytometry and confocal microscopy analyses.

2. Results and discussion

2.1. Design and synthesis of kisspeptin-based fluorescent probes for GPR54

Several fluorescent GPR54 probes **1-5** were designed based on Kp-52 and Kp-14 (Figure 1 and Scheme 1). During the design of these probes, the decision was taken to attach a fluorophore, such as tetramethylrhodamine (TMR) or rhodamine green (RG), to the N-terminus of the kisspeptins because the C-terminal sequence of these peptides is critical for their binding to GPR54.[11,13] The fluorophore group was directly attached to the N-terminus of Kp-52 and Kp-14. For the Kp-14-based probe **4**, a polyethylene glycol (PEG) linker was employed between the fluorophore and the peptide N-terminus to avoid the possibility of reduced binding affinity resulting from the bulky fluorophore group.[23]

Several TMR-labeled kisspeptin derivatives were synthesized. The peptide chains for Kp-52 and Kp-14 were constructed using Fmoc-based solid phase peptide synthesis (SPPS) on CLEAR-Amide

resin and NovaSyn TGR resin, respectively (Scheme 1). The TMR fluorophore and PEG linker were also introduced to the N-termini of the peptides on resin using carboxytetramethylrhodamine and Fmoc-NH-$(PEG)_2$-COOH, respectively. Treatment of the TMR-labeled protected peptide resins with TFA/thioanisole/*m*-cresol/1,2-ethanedithiol (EDT)/H_2O (16:1:1:1:1) gave Kp-14 derivatives **3** and **4**. TMR-labeled Kp-52 **1** was obtained by the deprotection and cleavage of the resin-bound Kp-52 derivatives followed by air oxidation. All of the TMR-labeled probes were purified by reverse-phase high performance liquid phase chromatography (RP-HPLC) and isolated as single regioisomers with regard to the fluorophore moiety (**1a**,**b**, **3a**,**b**, and **4a**,**b**).

The RG-labeled probes (**2** and **5**) were synthesized according to an alternative approach because the application of the on-resin labeling approach used for the TMR-labeled probes gave only low yields of the desired probes as well as significant quantities of byproducts. The RG-labeled probes were synthesized using a Hüisgen 1,3-dipolar cycloaddition reaction, which allowed for the fluorescent RG group to be introduced to the N-termini of the kisspeptins. Peptide chains containing an alkyne at their N-termini were constructed using Fmoc-based SPPS (Scheme 1), and the resulting protected peptide resins were treated according to the deprotection/cleavage conditions described above. Subsequent conjugation of the alkyne-containing kisspeptin derivatives with an RG-conjugated azide in the presence of $CuSO_4$, sodium ascorbate and tris(benzyltriazolylmethyl)amine (TBTA) gave the desired RG-labeled peptides Kp-52 **2** and Kp-14 **5**. It is noteworthy that the Cys4-Cys18 disulfide bond of **2** also formed under these conditions. The RG-labeled probes **2** and **5** were obtained as an inseparable mixture of regioisomers with regard to the fluorophore.

2.2. Biological evaluation of the fluorescent probes

The biological activities of fluorescent probes **1**–**5** towards GPR54 were investigated and the results are shown in Table 1. The binding activities were evaluated using a competitive inhibition experiment in GPR54-expressing Chinese hamster ovary cells (GPR54 CHO cells) with radiolabeled human kisspeptin-10 [metastin(45-54)]. The TMR- and RG-labeled Kp-52 probes (**1a,b** and **2**)

showed similar binding affinities to that of non-labeled Kp-52 towards GPR54 [IC_{50}(**1a**) = 3.4 nM, IC_{50}(**1b**) = 4.1 nM, IC_{50}(**2**) = 5.0 nM, IC_{50}(Kp-52) = 4.4 nM]. The Kp-14-based probes (**3a,b**, **4a,b** and **5**) showed slightly lower receptor binding affinities than the non-labeled Kp-14 [IC_{50}(Kp-14) = 1.5 nM], regardless of whether or not they contained a PEG linker [IC_{50}(**3a**) = 3.2 nM, IC_{50}(**3b**) = 5.4 nM, IC_{50}(**4a**) = 4.9 nM, IC_{50}(**4b**) = 7.4 nM, IC_{50}(**5**) = 3.1 nM]. These results indicate that the direct conjugation of the fluorophore group to the N-terminus of Kp-14 did not have a significant impact on the receptor binding. The receptor binding of the probes to GPR54 did not appear to be sensitive to the structures of the fluorophores or the different regioisomers of TMR.

We also assessed the GPR54-agonistic activity of TMR-labeled peptides by monitoring the intracellular Ca^{2+} flux in GPR54 CHO cells (Table 1).[12,24] The TMR-labeled Kp-52-based probes **1a,b** exhibited good GPR54 agonistic activities [EC_{50}(**1a**) = 46 nM, EC_{50}(**1b**) = 49 nM], although these values were two-fold greater than that of the parent Kp-52 [EC_{50}(Kp-52) = 23 nM]. The TMR-labeled Kp-14-based probes **3a,b**, and **4a,b** were three- to five-fold less potent than the parent Kp-14 [EC_{50}(Kp-14) = 12 nM] in terms of their GPR54 agonistic activity [EC_{50}(**3a**) = 36 nM, EC_{50}(**3b**) = 45 nM, EC_{50}(**4a**) = 60 nM, EC_{50}(**4b**) = 57 nM]. These results are therefore consistent with the receptor binding of the probes to GPR54.

We and others previously reported that short human kisspeptin peptides such as Kp-10 exhibited moderate binding activity, as well as the ability to activate neuropeptide FF receptors (i.e., human NPFFR1 and NPFFR2).[25,26] The selectivity profiles of rat Kp-52 and Kp-14 were investigated for rat NPFF receptors (i.e., rat NPFFR1 and NPFFR2) using competitive binding inhibition assays with radiolabeled NPFF (see Supplementary data). Rat Kp-52 and Kp-14 showed much lower inhibitory activities towards the NPFF receptors [IC_{50}(Kp-52) = 2.2 μM, IC_{50}(Kp-14) = 1.3 μM for NPFFR1; IC_{50}(Kp-52) = 2.2 μM, IC_{50}(Kp-14) = 1.8 μM for NPFFR2] compared with neuropeptide FF (NPFF), which was used as a control endogenous peptide for NPFF receptors [IC_{50}(NPFF) = 0.67 nM for NPFFR1; IC_{50}(NPFF) = 0.30 nM for NPFFR2].[27] Although the binding activities of the Kp-52- and Kp-14-based probes towards the NPFF receptors were low, the possibility of nonspecific binding to

these receptors could not be ruled out, especially when the probes were used at high doses.

2.3. Application of fluorescent probes to confocal microscopy and flow cytometry experiments

A confocal microscopy study using GPR54 CHO cells was conducted to demonstrate the applicability of the TMR-labeled (**1b** and **3b**) and RG-labeled (**2** and **5**) probes for the detection of GPR54 (Figure 2). When the GPR54 CHO cells were treated with these probes (500 nM) for 15 min, the fluorescent signals were observed in the intracellular compartments, which were accompanied by receptor internalization.[28,29] The staining of the GPR54-expressing cells was inhibited in the presence of non-labeled Kp-10 (10 μM). These results suggested that the fluorescent probes allowed for the specific detection of GPR54 whilst maintaining the bioactivity of the parent kisspeptins.

The RG-labeled probes **2** and **5** were used for the analysis of GPR54 CHO cells by flow cytometry (Figure 3). Treatment of the cells with the RG-labeled probes (100 nM) led to the occurrence of a fluorescent signal. No staining was observed in the GPR54-expressing cells when the same experiment was conducted in the presence of non-labeled Kp-10 (10 μM). Furthermore, the application of the RG-labeled probes to control cells not expressing GPR54 did not result in any staining (see Supplementary data). Taken together, these results suggested that the RG-labeled probes (**2** and **5**) were binding to GPR54 in a specific manner.

2.4. Intravenous injection of the TMR-labeled probe into male rats

A preliminary in vivo experiment was conducted using TMR-Kp-14 **3b**. Probe **3b** (10 nmol/kg) was intravenously injected into male rats, and the plasma LH concentration was subsequently measured by a double-anti-body radioimmunoassay (RIA).[5] The plasma LH level increased at 6 and 12 min after the injection of **3b** (Figure 4), which was consistent with the results obtained in our previous study using unlabeled Kp-10.[5] This result suggested that TMR-Kp-14 **3b** was a useful probe, and that it possessed the necessary GPR54 agonistic activity to promote LH secretion in an in vivo experiment. Further study aimed at investigating the distribution of a series of probes as well as the

expression and location of GPR54 are currently underway.

3. Conclusions

In conclusion, we have designed and synthesized several fluorescent probes for rat GPR54, which were based on the endogenous rat kisspeptins, Kp-14 and Kp-52. The newly synthesized TMR- and RG-labeled kisspeptins exhibited comparable levels of biological activity to the parent unlabeled kisspeptins when they were evaluated in in vitro GPR54 binding and activation experiments. Confocal microscopy analysis suggested that the probes could be used to trace the receptor internalization of GPR54-expressing cells. The RG-labeled probes were also suitable for the analysis of GPR54-expressing cells by flow cytometry. Furthermore, the TMR-labeled peptide effectively induced the release of LH following its peripheral administration in rats. The results of the current study demonstrate that our labeled peptides could be used as selective molecular probes for the GPR54 receptor in in vitro and in vivo experiments.

4. Experimental

4.1. Synthesis

4.1.1. General methods

Analytical HPLC was performed using a Cosmosil 5C18-ARII column (4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan). The column was eluted with a linear gradient of CH_3CN containing 0.1% (v/v) TFA at a flow rate of 1 mL/min, and the products were detected by UV at 220 nm. Preparative HPLC was performed using a Cosmosil 5C18-ARII column (20 × 250 mm or 10 × 250 mm, Nacalai Tesque). The column was eluted with a linear gradient of CH_3CN containing 0.1 % (v/v) TFA at a flow rate of 8 or 4 mL/min, respectively. The peptides were characterized by MALDI-TOF-MS (AXIMA-CFR plus, Shimadzu) (see Supplementary data). The compound purities were determined to be greater than 95% by analytical HPLC.

4.1.2. Preparation of Kp-52 peptides (1a,b)

The Kp-52 peptide chain was constructed by Fmoc-based SPPS on CLEAR-Amide resin (0.4 mmol/g, 50 mg, 0.02 mmol) using a PSSM-8 automatic peptide synthesizer (Shimadzu). *t*-Bu ester for Asp and Glu; *t*-Bu for Ser, Thr and Tyr; Boc for Lys; Trt for Cys, His, Asn and Gln; and Pbf for Arg were employed for side-chain protections. The Fmoc-protected amino acids (0.10 mmol) were coupled using a mixture of *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU) (37.9 mg, 0.10 mmol), 1-hydroxybenzotriazole hydrate (HOBt·H_2O) (15.3 mg, 0.10 mmol) and (*i*-Pr)$_2$NEt (34.8 μL, 0.20 mmol) in DMF (or DMF/NMP for Fmoc-Phe-OH) over 45 min. This process was performed twice. Subsequent treatment with 20% piperidine in DMF allowed for the cleavage of the Fmoc-protecting groups from the resin. Carboxytetramethylrhodamine (25.8 mg, 0.06 mmol) or 5-hexynoic acid (11.2 mg, 0.10 mmol) was manually coupled to the peptide resin (0.02 mmol) using a mixture of *N*,*N*′-diisopropylcarbodiimide (DIC) and HOBt·H_2O in DMF. The resulting protected peptide resin was treated with TFA/thioanisole/*m*-cresol/1,2-ethanedithiol/H_2O (16:1:1:1:1) at room temperature for 2 h. The resin was then removed by filtration, and the resulting filtrate was treated with ice-cold anhydrous Et_2O, which resulted in the precipitation of a powder. The powder was collected by centrifugation and then washed three times with ice-cold anhydrous Et_2O. The crude product was diluted with water (pH 7.9-8.1), and then incubated at room temperature in an open vessel for a few days to allow for aerial oxidation. The crude peptide was purified by preparative HPLC to give the desired peptides **1a,b** as powders (single fluorophore-regioisomers).

4.1.3. Preparation of Kp-14 peptides (3a,b and 4a,b)

The protected peptide resin for Kp-14 was manually constructed by Fmoc-based SPPS on NovaSyn TGR resin (0.26 mmol/g, 384 mg, 0.10 mmol) using a mixture of DIC (0.0464 mL, 0.30 mmol) and HOBt·H_2O (45.9 mg, 0.30 mmol) in DMF. *t*-Bu ester for Asp; *t*-Bu for Ser and Tyr; Trt for Asn; and Pbf for Arg were employed for side-chain protections. Fmoc-NH-(PEG)$_2$-COOH (20 atoms, 168 mg, 0.30 mmol, Novabiochem), carboxytetramethylrhodamine (129 mg, 0.30 mmol) and

5-hexynoic acid (56.1 mg, 0.50 mmol) were also coupled to the peptide resin (0.10 mmol) using a mixture of DIC and HOBt·H_2O in DMF. The subsequent deprotection and purification processes were performed according to the procedures described above to give the desired peptides **3a,b** or **4a,b** as powders (single fluorophore-regioisomer).

4.1.4. Conjugation of rhodamine green (RG): synthesis of RG-Kp-52 (2)

$CuSO_4$ in H_2O (0.2 M, 0.020 mL, 4 μmol) and tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) in DMSO (0.2 M, 0.020 mL, 4 μmol) were added to H_2O-DMSO-*t*-BuOH (21:7:40, 0.46 mL) and the solution was stirred for 3 min. Then, a solution of sodium ascorbate (0.2 M, 0.040 mL, 8 μmol) was slowly added to the mixture. This reagent cocktail was added to the mixture of alkyne-containing Kp-52 (300 nmol) and RG-azide (1 μmol) in DMSO (0.10 mL). After being stirred at room temperature for 10 min, the mixture was purified by preparative HPLC to provide RG-Kp-52 (0.82 mg, 111 nmol, 37%) as freeze-dried powder (mixture of fluorophore-regioisomers). RG-Kp-14 (**5**) was synthesized essentially in the same manner.

4.2. Biological evaluations

4.2.1. Cell lines

For generation of the cDNA for the rat GPR54, a *Hind III* site was inserted at the 5′ site of the GPR54 using GPR54/F (TATAAAGCTTATGGCCGCAGAGGCGACGTT) as a forward primer and a *Xho I* site was inserted at the 3′ site of the GPR54 using GPR54/R (TATACTCGAGTCAGAGTGGGGCAGTGTGTT) as a reverse primer, respectively. The resulting PCR product was digested with *Hind III* and *Xho I*, and inserted into the *Hind III*/*Xho I* site of pcDNA5/FRT/TO, to obtain pcDNA5/FRT/TO-rGPR54.

Flp-In CHO cells (Invitrogen) were maintained in Ham's F12 medium (Wako) with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin in 5% CO_2 at 37 °C. The GPR54 constructs were cotransfected with pOG44 (Invitrogen), an expression vector for

Flp recombinase, into Flp-In CHO cells according to the manufacturer's protocol. The cells were cultured in the medium in the presence of 500 μg/mL hygromycin. The cells that were resistant to hygromycin were then selected.

4.2.2. Evaluation of the binding affinity to GPR54

The binding affinities of the probes for GPR54 were evaluated using the membrane fraction of GPR54 CHO cells with [^{125}I]-metastin 45-54 (PerkinElmer Life Sciences), which was used as a radioactive. Samples (25 μL) of a membrane solution in assay buffer [50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM EGTA, 0.5% BSA] were incubated with 50 μL solutions of the different fluorescent probe and 25 μL of [^{125}I]-metastin 45-54 (0.2 nM) at room temperature for 1 h. The reaction mixtures were then filtered through GF/B filters (PerkinElmer Life Sciences), which had been pretreated with 0.3% polyethyleneimine. The filters were then washed with a buffer [10 mM HEPES (pH 7.4), 500 mM NaCl] and then dried at 55 °C. The bound radioactivity was measured by TopCount (PerkinElmer Life Sciences) in the presence of MicroScint™-O (30 μL).

4.2.3. Evaluation of the GPR54 agonistic activity of synthesized probes

The GPR54 agonistic activities of the newly synthesized probes were evaluated by monitoring intracellular Ca^{2+} flux levels in rat GPR54 CHO cells. GPR54 CHO cells (4.0×10^4 cells/50 μL/well) were inoculated in 10% FBS/Ham's F-12 medium on a 96-well plate (black clear-bottom plate, Greiner), and incubated in 5% CO_2 at 37 °C for 24 h. The medium was then removed from the plate and replaced with 100 μL of the pigment mixture (Calcium 4 assay kit, Molecular Devices), which was dispensed into each well of the plate. The resulting mixtures were then incubated in 5% CO_2 at 37 °C for 1 h. Samples of the peptide in DMSO (10 mM) were diluted with HANKS/HEPES containing 2.5 mM probenecid and transferred to a 96-well sample plate (V-bottom plate, Coster). The cell and sample plates were then placed in a FlexStation (Molecular Devices) and 25 μL portions

of the sample solutions were automatically transferred to the cell plate. Time dependent changes in the intracellular calcium ion levels were then measured.

4.2.4. Confocal microscopy analysis

GPR54 CHO cells were plated on 35 mm glass-bottomed dishes and cultured in 10% FBS/Ham's F-12 medium. The cells were washed three times with PBS (+) and incubated with each fluorescent peptide (500 nM) in the presence or absence of unlabeled rat Kp-10 (10 μM) at 37 °C for 15 min. After being rinsed three times with PBS (+), the cell were observed by confocal microscopy using an Eclipse Ti-E confocal microscope (Nikon). The green (RG) channel was excited by a 488 nm laser and detected through a BP 500−550 nm emission filter. The red (TMR) channel was excited by a 561 nm laser, and detected through a BP 575−615 nm emission filter.

4.2.5. Flow cytometry analysis

The GPR54 CHO cells were detached using versene and the resulting cells (5.0×10^5 cells) were resuspended in 100 μL of a probe solution [i.e., 100 nM of the RG-labeled probe in PBS (0.1% BSA)]. After being incubated at 37 °C for 15 min, the cells were washed with PBS (0.1% BSA) and the fluorescence intensity was analyzed using a FACSCalibur system (BD Biosciences). The resulting data were collected from FL1 in the log mode and the fluorescent intensity values were calculated as the geometric mean of cellular fluorescence. Data were analyzed using *CellQuest Pro* software (BD Biosciences).

4.2.6. Injection of TMR probe into rat and subsequent monitoring of plasma LH level

An animal experiment was conducted in accordance with the guidelines of the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University. TMR-Kp-14 (**3b**) was injected (10 nmol/kg) into the jugular vein of freely-moving conscious male rats (12 weeks old, $n = 2$) through a silicon cannula (0.5-mm inner diameter; 1-mm outer diameter; Shin-Etsu

Polymer) that had been inserted into the right atrium on the day before blood sampling. Blood samples were collected from the rats through the cannula just before the injection of the TMR probe, and both 6 and 12 min after the injection. The plasma LH concentrations were determined by a double-antibody radioimmunoassay (RIA) according to a previously published procedure.[5]

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27. The low binding affinity of rat kisspeptins to NPFF receptors may be derived from the different C-terminal residue (Phe in primates, Tyr in others).

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29. Of note, the localization patterns of TMR-labeled probes (**1b** and **3b**) and RG-labeled probes (**2** and **5**) were different. A portion of TMR-labeled probes were apparently translocated into a specific region by an unidentified process. This translocations were also inhibited in the presence of unlabeled Kp-10.

Figure 1. Design of the Kp-52- and Kp-14-based fluorescent probes for GPR54.

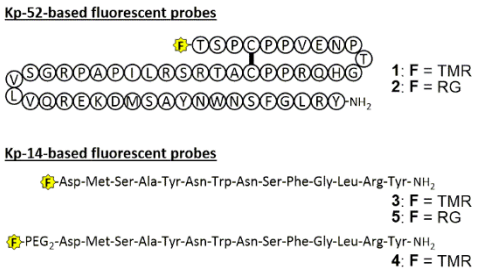


Figure 2. Confocal microscopy images of GPR54-expressing CHO cells stained with the fluorescent probes. The cells were incubated with TMR-Kp-14 **3b** (500 nM) in the absence or presence of non-labeled Kp-10 (10 μM) (a). GPR54-expressing cells were treated with RG-Kp-14 **5** (b), TMR-Kp-52 **1b** (c) and RG-Kp-52 **2** (d) (500 nM).

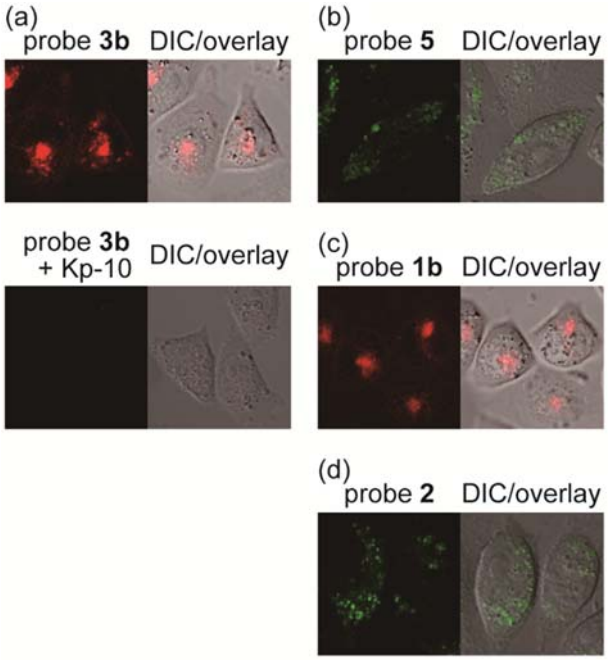


Figure 3. Application of RG-labeled probes to flow cytometry. GPR54-expressing CHO cells were treated with RG-Kp-52 **2** (A) and RG-Kp-14 **5** (B) (100 nM). The upper panels show the results in the absence of non-labeled Kp-10 and the lower panels show the results in the presence of non-labeled Kp-10 (10 μM).

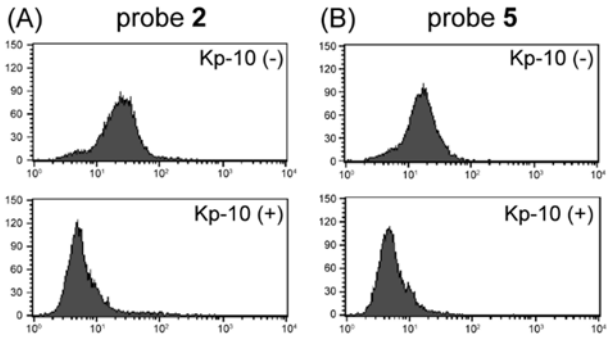
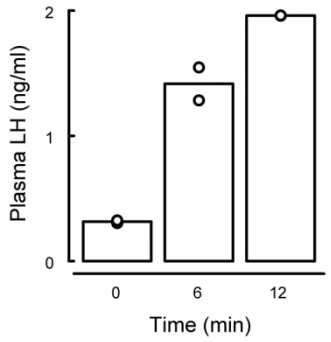


Figure 4. Effect of the intravenous administration of TMR-Kp-14 **3b** on the plasma LH levels in male rats (12 weeks old). Plasma samples were collected at 0 (just before injection), 6 and 12 min after the intravenous injection of **3b** (10 nmol/kg). The values are the mean plasma LH levels (n = 2). The open circles indicate individual plasma LH levels.



Scheme 1. Synthesis of fluorescent kisspeptin-based probes. (A) Kp-52-based probes. (B) Kp-14-based probes. Reagents and conditions: (a) Fmoc-based solid-phase peptide synthesis; (b) TFA/H_2O/1,2-ethanedithiol/*m*-cresol/thioanisole (16:1:1:1:1); (c) air oxidation; (d) RG-N_3, $CuSO_4$, sodium ascorbate, TBTA.

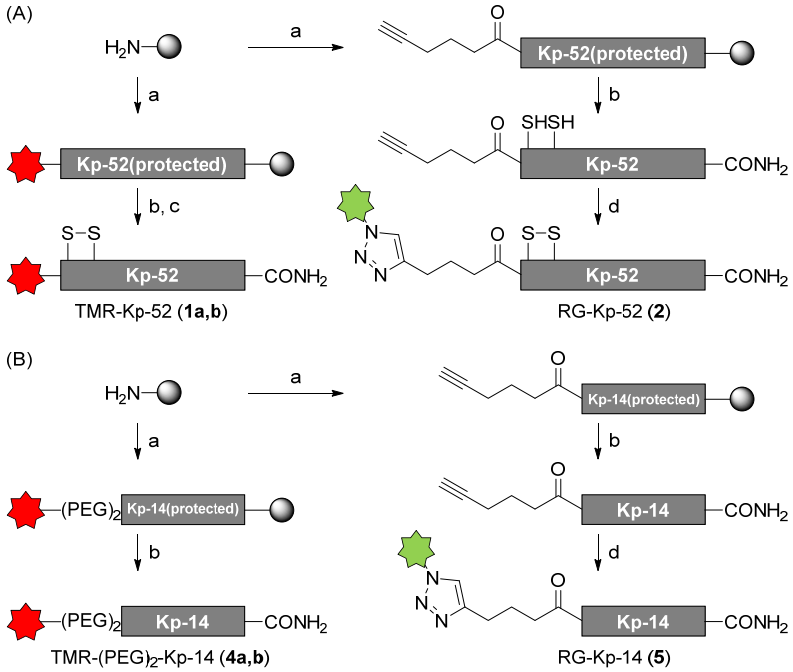


Table 1. Biological activities of probes **1**–**5**.

Probe	Fluorophore	Linker	Sequence	IC_{50} (nM)[a]	EC_{50} (nM)[b]
Kp-52				4.4	23
1a[c]	TMR	-	Kp-52	3.4	46
1b[c]	TMR	-	Kp-52	4.1	49
2[d]	RG	-	Kp-52	5.0	-[e]
Kp-14				1.5	12
3a[c]	TMR	-	Kp-14	3.2	36
3b[c]	TMR	-	Kp-14	5.4	45
4a[c]	TMR	$(PEG)_2$	Kp-14	4.9	60
4b[c]	TMR	$(PEG)_2$	Kp-14	7.4	57
5[d]	RG	-	Kp-14	3.1	-[e]
Kp-10				0.60	11

[a] IC_{50} values were calculated from the dose–response curves generated from triplicate data points in competitive experiment with [^{125}I]Tyr-metastin(45-54). [b] EC_{50} values were calculated from the dose–response curves generated from triplicate data points in $[Ca^{2+}]_i$ flux assay. [c] Single fluorophore-regioisomer. [d] Mixture of fluorophore-regioisomers. [e] Not determined.