博士論文

Design and Synthesis of Short-length KISS1R Agonists for the Functional Analysis of an Orphan G Protein–coupled Receptor KISS1R

（オーファン受容体 **KISS1R** の機能解析を指向した
短鎖型 **KISS1R** リガンドの創製）

浅見　泰司

Contents

Chapter 1 General Introduction

1.1. Significance of G Protein–coupled Receptors (GPCRs) as Drug Targets.

G protein–coupled receptors (GPCRs) constitute the largest family of membrane proteins and play pivotal roles in a diverse range of biological functions in living cells. There is a high proportion of drugs targeting GPCRs when comparing the number of launched and clinical drugs in terms of target protein class (**Figure 1**).[1]

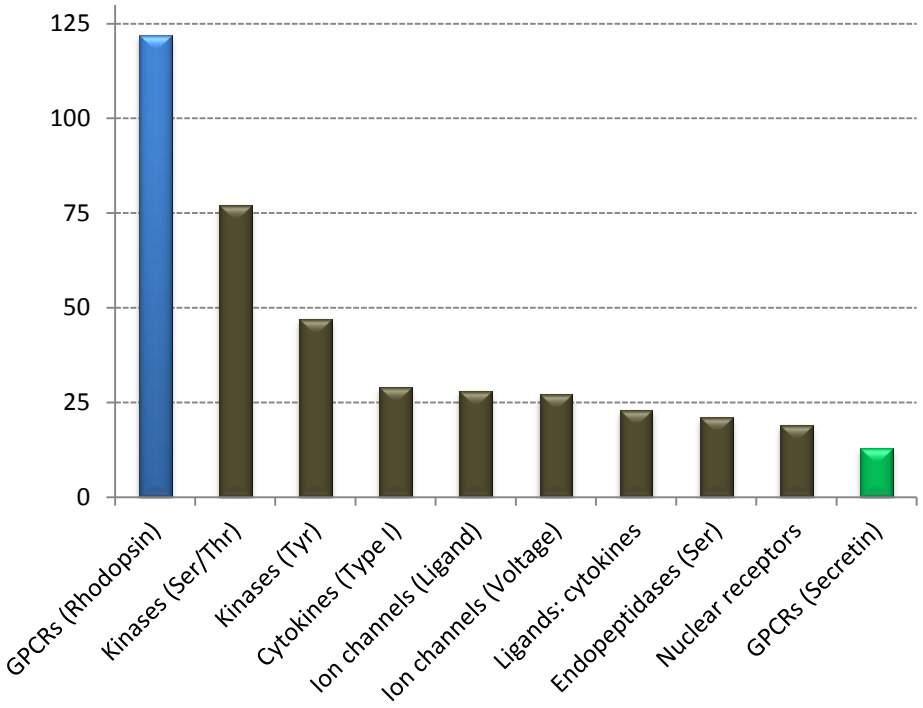


Figure 1. Top 10 target families of established and clinical trial drug targets. This figure is created by the data of *Annu. Rev. Pharmacol. Toxicol.* **2014**, *54*, 9-26.

It is quite obvious that there is a great importance to the drugs targeting GPCRs. There are nearly a thousand different GPCRs in the human genome and a lot of orphan GPCRs without a endogenous ligand are remained to be coded.[2] However, novel endogenous ligands for more than 70 orphan GPCRs have been identified in recent world-wide studies (**Figure 2**).

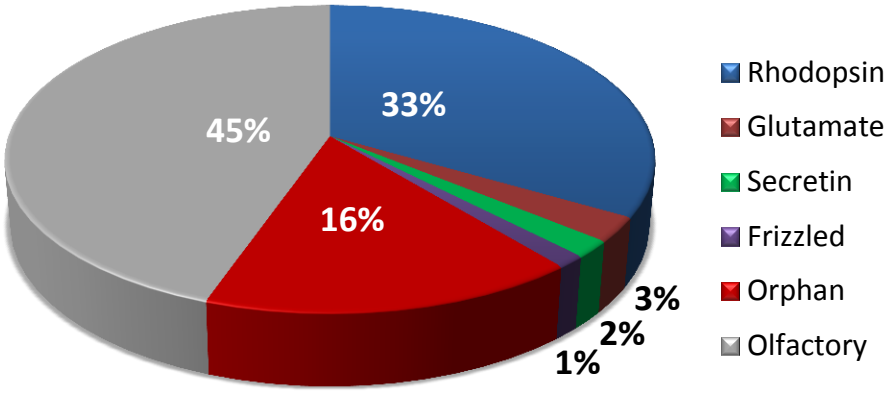


Figure 2. Ratio of GPCRs classified based on the GRAFS system. This figure is created by the data of *Annu. Rev. Pharmacol. Toxicol.* **2013**, *53*, 127-46.

1.2. Roles of Metastin and KISS1 Receptor.

1.2.1. About Human Metastasis Suppressor Gene *KISS1*.

Novel key genes expressed in both human placenta and malignant tumors have been identified and implicated in the modulation of tumor invasion and metastasis. In this regard, *KISS1* has been identified as a human metastasis suppressor gene encoding a *C*-terminal amidated protein, metastin, that suppresses metastasis of human melanomas and breast carcinomas.[3-5]

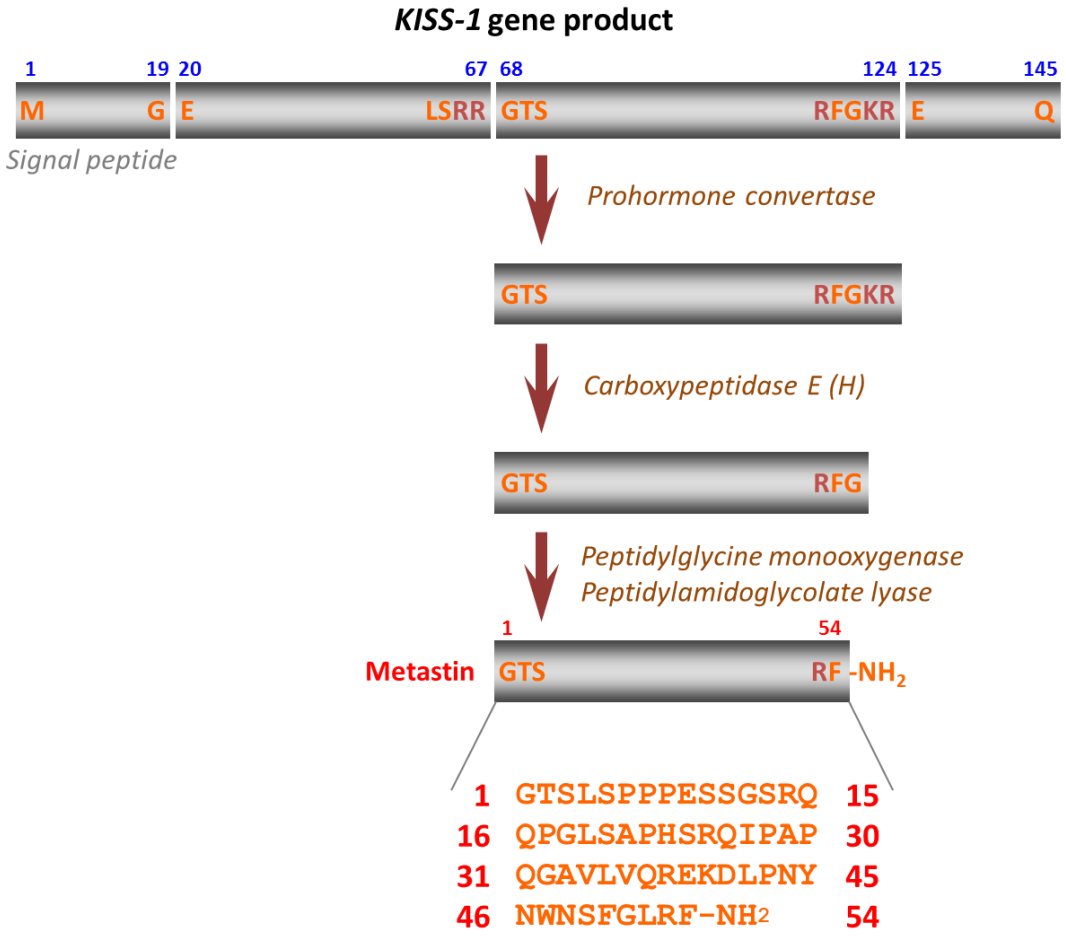


Figure 3. Hypothetical processing pathway of *KISS-1* gene product including removal of the signal peptide and cleavage by a prohormone convertase and a carboxypeptidase.

Metastin/kisspeptin was first isolated from human placenta and proposed to be the

endogenous ligand of an orphan G-protein-coupled receptor KISS1R, also known as GPR54 or AXOR12, and recently renamed KISS1R that is expressed throughout the central nervous system and in a variety of endocrine and gonadal tissues.[3-5]

Metastin, which is an amidated peptide with 54 amino acid residues, is encoded by the metastasis-suppressor (*KISS1*) gene, the mRNA of which is expressed in the human placenta and brain, as well as in rodents (**Figure 3**). Therefore, metastin was firstly thought to facilitate the suppression of metastasis. Actually, metastin activates KISS1R and inhibits chemotaxis, cell motility and growth, and metastasis of human KISS1R (hOT7T175)-transfected B16-BL6 melanomas *in vivo*.[3-8] However, physiological function of metastin as the metastasis suppressor has not been well elucidated.

1.2.2. Metastin as a Critical Regulator of Gonadotropin Releasing Hormone (GnRH) Secretion.

More recently, fundamental studies have suggested that metastin acts as a critical regulator of gonadotropin releasing hormone (GnRH) secretion. Indeed, single administration of metastin potently induces follicle stimulating hormone (FSH)/ luteinizing hormone (LH) release in several mammalian species including rats (**Figure 4**),[9] mice,[10] and human males.[11]

Detailed observations revealed that *KISS1* mRNA is expressed in the hypothalamic brain region,[10] and KISS1R mRNA is expressed in hypothalamic GnRH neurons,[12] suggesting that metastin directly regulates GnRH release from GnRH neurons. The mutation of KISS1R gene was identified as a causative gene of idiopathic hypogonadotropic hypogonadism (IHH).[13]

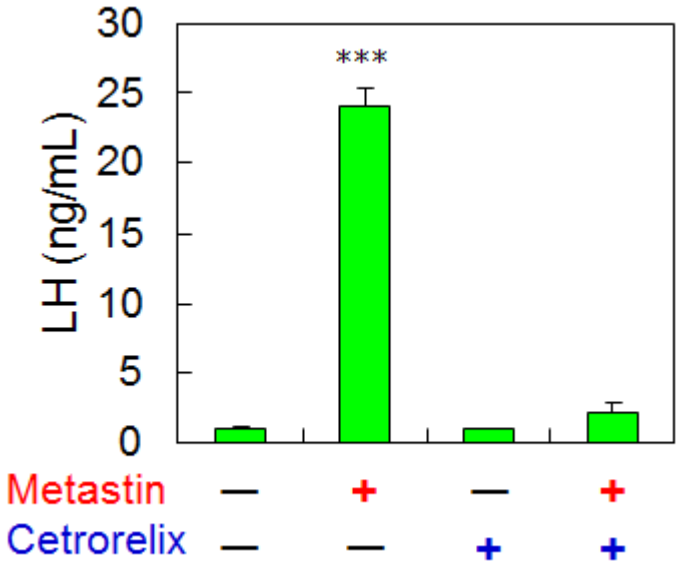


Figure 4. The effect of metastin and cetrorelix on gonadotropin release in adult male Wistar rats (n = 5). Plasma LH levels in 10-week-old rats 90 min after subcutaneous administration of either metastin (100 nmol/kg) or saline, following pretreatment with cetrorelix (600 nmol/kg) or saline. Bars show means+SEM. ***P < 0:001 versus the other three groups by Tukey's test. This data was published in *Biochem Biophys Res Commun* **2004,** *320,* 383-8.

1.2.3. Phenotype of KISS1R Knockout Mice.

Furthermore, KISS1R knockout mice showed a phenotype similar to human IHH.[14-15] The hypothalamic–pituitary–gonadal (HPG) axis is well known to regulate reproduction in all vertebrates by controlling the uterine and ovarian cycles; these findings indicated that metastin functions in the HPG axis (**Figure 5**). Taking this opportunity, physiological roles of metastin and KISS1R received a lot of attention in the field of neuroendocrinology.

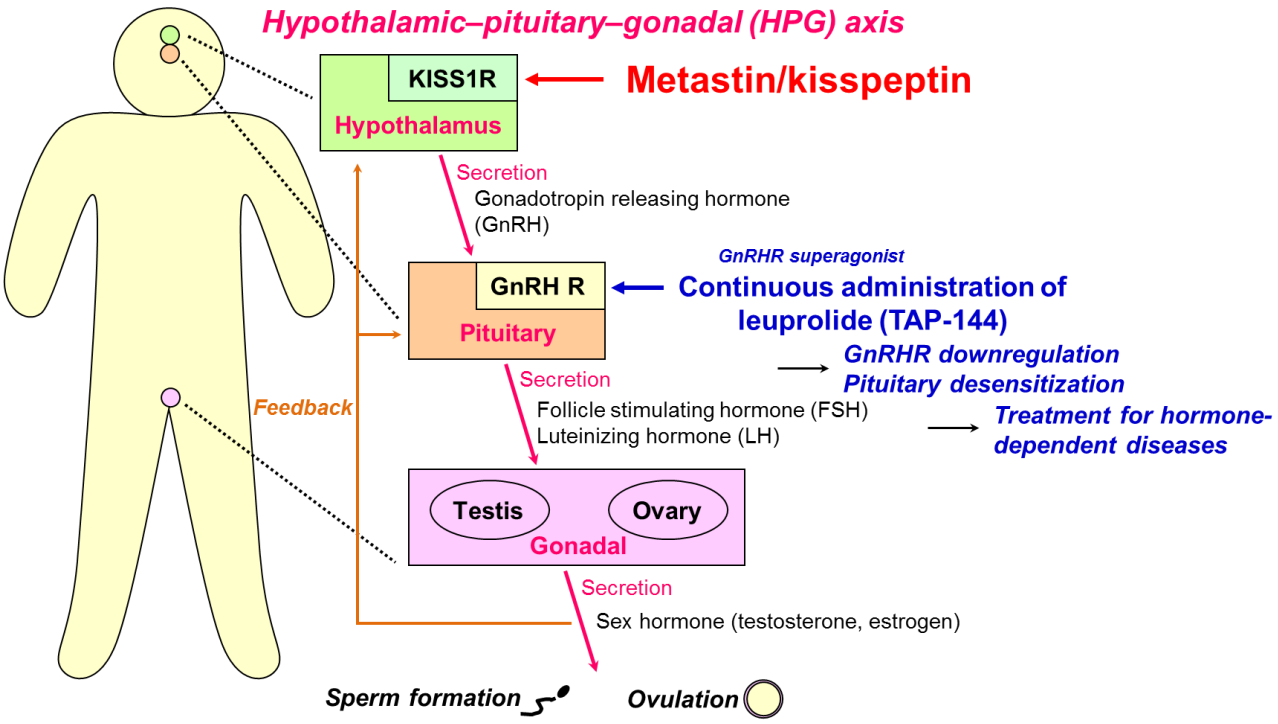


Figure 5. Schematic illustration demonstrating HPG axis, which regulate reproduction in all vertebrates.

1.2.4. Hypothalamic–Pituitary–Gonadal (HPG) Axis.

Hypothalamic hormones are synthesized by neurons in the hypothalamus and secreted into hypophyseal portal system to regulate the synthesis and release of anterior pituitary hormones. Hypothalamic hormones are divided into two classes, pituitary hormone releasing hormones and inhibitory hormones. More than ten hypothalamic hormones are thought to exist; six of them are identified by chemical structures and physiological roles. GnRH is one of hypothalamic hormones and composed of ten amino acids which stimulate the synthesis and release of LH and FSH (**Figure 5**). Gonadotropins are controlled by GnRH via a G protein-coupled receptor that activates phospholipase C transmiting its signal to diacylglycerol (DAG) and inositol

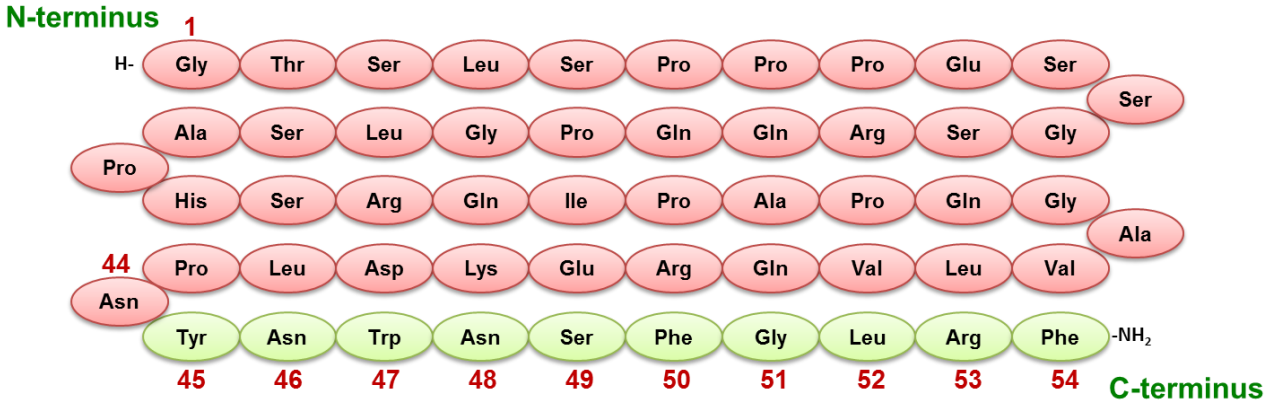
1,4,5-trisphosphate (IP3). The gonadotropins stimulate the gonads, ovary, and testis, for follicular growth, ovulation, luteinization and sperm formation. Furthermore, the gonadotropins produce the sex steroids, estrogens and androgens, respectively.

1.2.5. Pharmaceutical Target.

Thus, drugs regulating the secretion of the gonadotropins have been thought to have therapeutic potentials for prevention or treatment of sex hormone dependent diseases, such as prostate cancer, gynecological disorder, infertility. There have been tremendous research and development activities in the past decades to discover that continuous administration of GnRH agonist induces downregulation and desensitization of GnRH receptor in the pituitary, which causes the suppression of gonadotropin release and the decrease in testosterone levels. By use of the findings, GnRH agonists such as leuprolide are widely used clinically for the treatment of hormone-dependent prostate cancer, breast cancer, endometriosis, uterine fibroid and precocious puberty. On the other hand, GnRH antagonists have been developed for the avoidance of the initial increase in gonadotropin release and gonadal hormone secretion after dosing of GnRH agonists, so-called "flare up" with this medication.

1.3. Active Site of Endogenous Ligand Metastin.

Initial research revealed that the *N*-terminal truncated human metastin(45-54), Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2, not only has the complete biological activity of the full metastin protein but is 3 to 10 times more active than metastin (**Figure 6**).[3]



	agonist activity (FLIPR [a])	binding affinity
	EC_{50} (nM)	IC_{50} (nM) [b]
metastin	**0.2**	**0.3**
metastin(45-54)	**0.01**	**0.04**

[a] FLIPR: Fluorescent Imaging Plate Reader; [b] IC_{50} values: peptide concentrations leading to 50 % inhibition in competitive binding analysis using 100 pM [^{125}I-Tyr^{45}]metastin(40-54).

Figure 6. Structures and biological activities of metastin and metastin(45-54) described in *Nature* **2001**, *411*, 613-617.

Although short-length analogues of metastin have the potential of research tool for the elucidation of metastin and KISS1R functions and possess therapeutic properties, their susceptibility to enzyme cleavage limits their utility.[16] Generally, peptides are degraded by proteases in serum, especially short-length peptides show rapid enzymatic degradation.

1.4. Aims of This Study.

The previous results showing the proposed function of metastin at the upper stream of GnRH suggest that appropriate dosing of KISS1R agonists can manipulate the hypothalamic-pituitary-gonadal axis with the potential to assist in the prevention or treatment of a number of sex hormone-dependent diseases.

In this study biological stability of metastin(45-54) was planned to evaluate for the rational synthetic strategy to give enhanced biological stability of short-length KISS1R agonists with the maintenance of potent agonist activities. One of the aims of this research was to obtain tool peptides capable of working *in vivo* to elucidate physiological functions of metastin and KISS1R (**Figure 7**).

- Discovery of tool peptide working in vivo to elucidate physiological functions of metastin and KISS1R.

[Synthetic strategy]

- Design and synthesis of short-length metastin(45-54) analogues with the maintenance of KISS1R agonist activity and improved biological stability.

[Targeted profiles]

- Molecular weight: less than one-fifth of metastin (54 amino acids)
- KISS1R affinity: more potent than metastin
- KISS1R agonist activity: more potent than metastin
- Biological stability: more than 50% residual rate in rodent serum for 60 min at 37°C
- Water solubility: more than 1 mM

- Establishment of general strategy for the drug discovery and development targeting orphan GPCRs by using biologically stable peptide analogue.

Figure 7. Aims of this study about design and synthesis of short-length KISS1R agonist.

Tool peptides were thought to be designed and synthesized using appropriate amino acid substitutions and amide bond isosteres to maintain KISS1R agonist activity and avoid degradation by proteases. Target profiles of tool peptides were set in terms of molecular weight, KISS1R affinity, KISS1R agonist activity, biological stability and water solubility. Molecular weight with less than one-fifth of metastin (54 amino acids), KISS1R affinity with more potent than metastin, KISS1R agonist activity with more potent than metastin, biological stability with more than 50% residual rate in rodent serum for 60 min at 37°C and water solubility with more than 1 mM were supposed to be needed for tool peptides working *in vivo*.

Moreover, the other aim of this research was to establish general strategy for the drug discovery and development targeting orphan GPCRs by using biologically stable peptide analogue. Biologically stable peptide was thought to be a powerful tool to elucidate functions of orphan GPCRs with peptide ligands.

This thesis presents rational strategies to give novel KISS1R agonists and a new insight into drug discovery and development targeting orphan GPCRs with peptide ligands.

Chapter 2 Trypsin Resistance of a Decapeptide KISS1R Agonist Containing an N^{ω}-Methylarginine Substitution

2.1. Summary of Chapter 2.

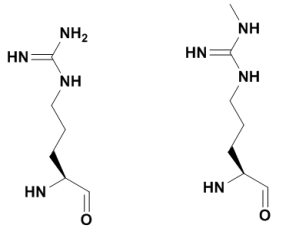
Metastin/kisspeptin is an amidated peptide with 54 amino acid residues; compared to the full-length metastin, the *N*-terminal truncated peptide metastin(45-54) has 3–10 times higher receptor affinity and enhanced ability to increase intracellular calcium concentration which is essential for activation of protein kinases involved in intracellular signaling in a number of pathways that affect reproduction and cell migration. However, metastin(45-54) is rapidly inactivated in serum. In this chapter, we designed and synthesized a number of metastin(45-54) analogues and evaluated their agonist activity and trypsin resistance. Among analogues with substitutions of arginine at position 53, N^{ω}-methylarginine (Arg(Me)) analogue **8** showed 3-fold more potent agonist activity compared with metastin(45-54). Furthermore, analogue **8** was shown to resist trypsin cleavage between positions 53 and 54. This substitution may be useful in the development of other Arg-containing peptides for which the avoidance of cleavage is desired.

Metastin(45-54)

Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg^{53}-Phe^{54}-NH_2

Compound **8**

Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg(Me)^{53}-Phe^{54}-NH_2



Arg Arg(Me)

2.2. Background and Strategy.

Native metastin consists of 54 amino acid residues. However, the *N*-terminal truncated human metastin(45-54), Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2, not only has the complete biological activity of the full metastin protein but is 3 to 10 times more active than metastin.[3] Although short-length analogues of metastin have the potential to possess therapeutic properties, their susceptibility to enzyme cleavage is thought to limit their utility.[16] Biological stability of metastin(45-54) has not been investigated in detail and the cleavage sites by serum proteases have not been well elucidated. In this chapter biological stability of metastin(45-54) was planned to evaluate for the rational synthetic strategy to give enhanced biological stability of short-length KISS1R agonists with the maintenance of potent agonist activities. Cleavage sites of metastin(45-54) by proteases were planned to identify by LC/MS/MS methodology after the treatment of mouse plasma. Subsequent amino acid substitutions and/or incorporation of amide bond isosteres at cleavage sites were thought to be resistant to serum proteases. All analogues were designed as ten-amino-acid peptides and evaluated in terms of KISS1R agonist activity, followed by the selection of potent analogues for the further investigation of biological stability using biological fluid or protease.

2.3. Determination of Biological Stability of Metastin(45-54).

First, the biological stability of short-length peptide metastin(45-54) was investigated; metastin(45-54) was rapidly metabolized in mouse plasma, and more than 50% of the peptide was metabolized after a 1-min incubation at 37°C (**Figure 8**).

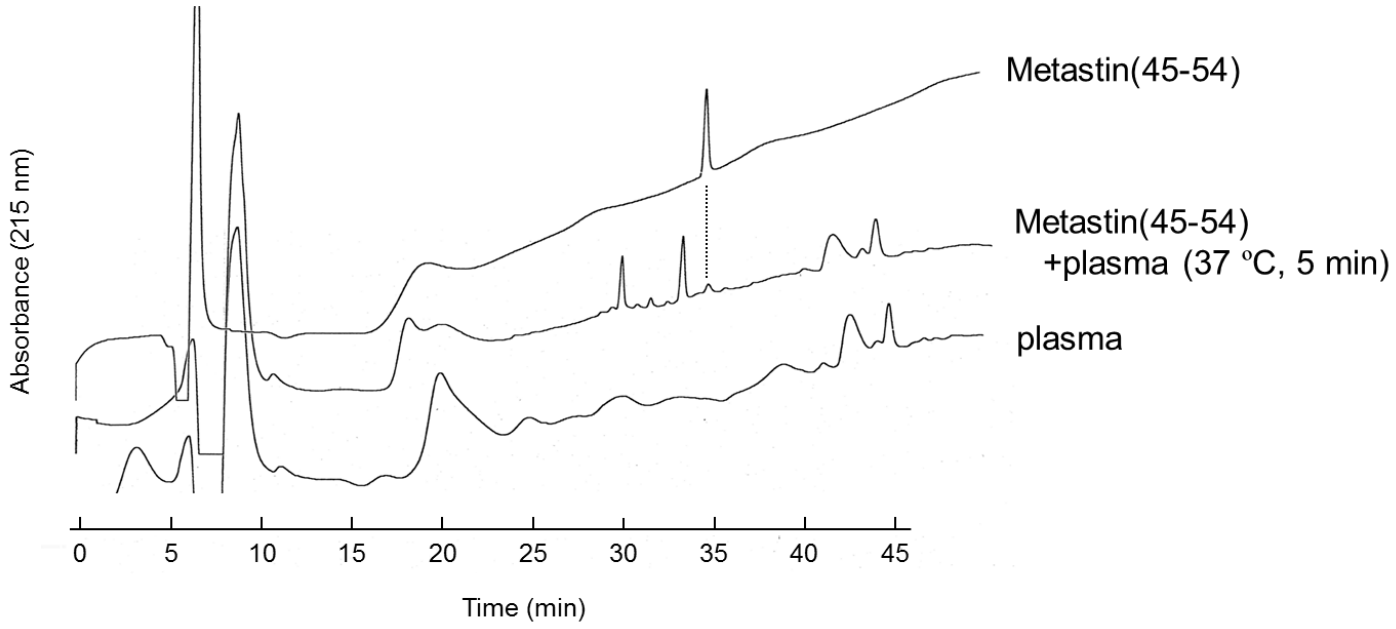


Figure 8. HPLC chromatogram of metastin(45-54) after incubation in mouse plasma.

The metabolites formed during the incubation in serum were identified by high-performance liquid chromatography/electrospray ionization mass spectrometry (HPLC-ESI-MS) coupling experiments using an ESI ion trap mass spectrometer (LCQ, Thermo-Fisher) (**Figure 9**).

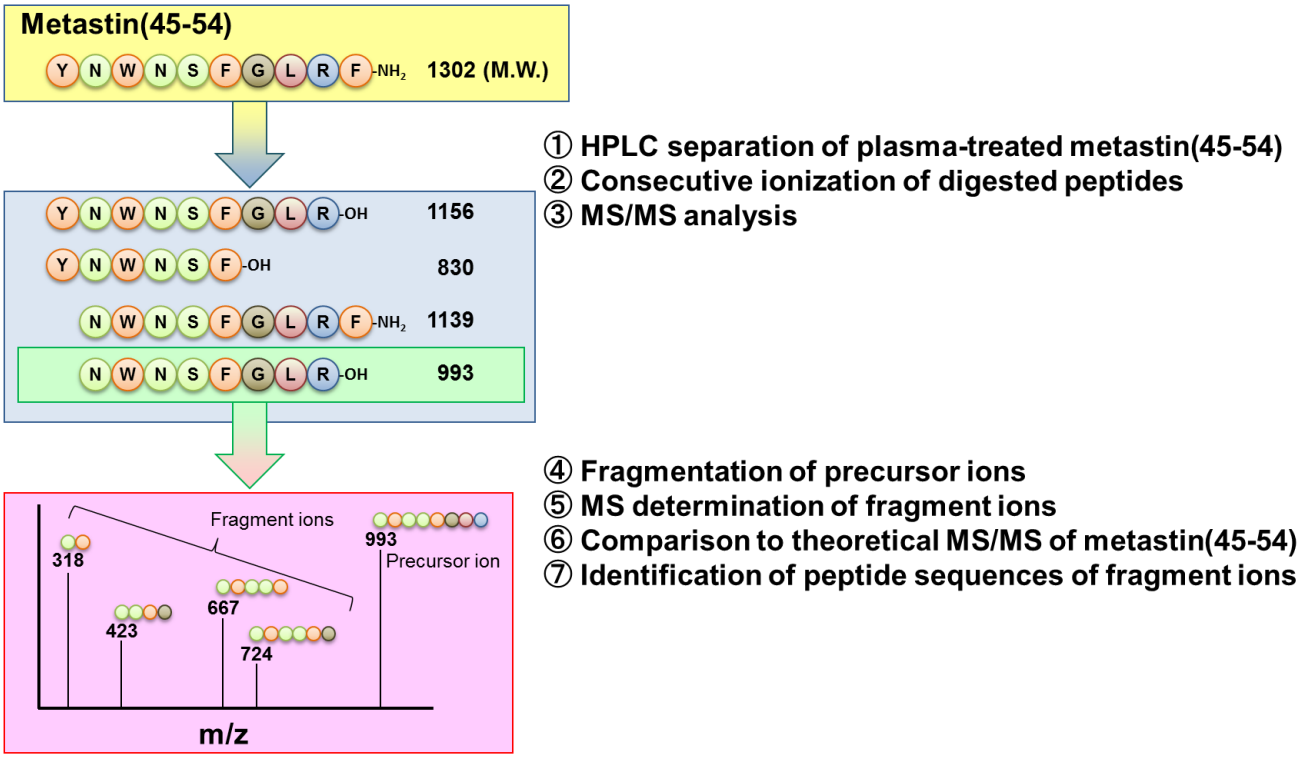


Figure 9. Schematic diagram to analyze digested peptides of metastin(45-54) by HPLC-ESI-MS coupling experiments.

ESI mass spectra obtained from metastin(45-54) incubated with mouse plasma revealed the presence of more than ten plausible metabolites (**Figure 10**). Two major peptide fragments, metastin(46-54) and (46-53), were released primarily as a result of Tyr^{45}-Asn^{46} and Arg^{53}-Phe^{54} protease cleavage due to aminopeptidase activity and trypsin-like degradation.

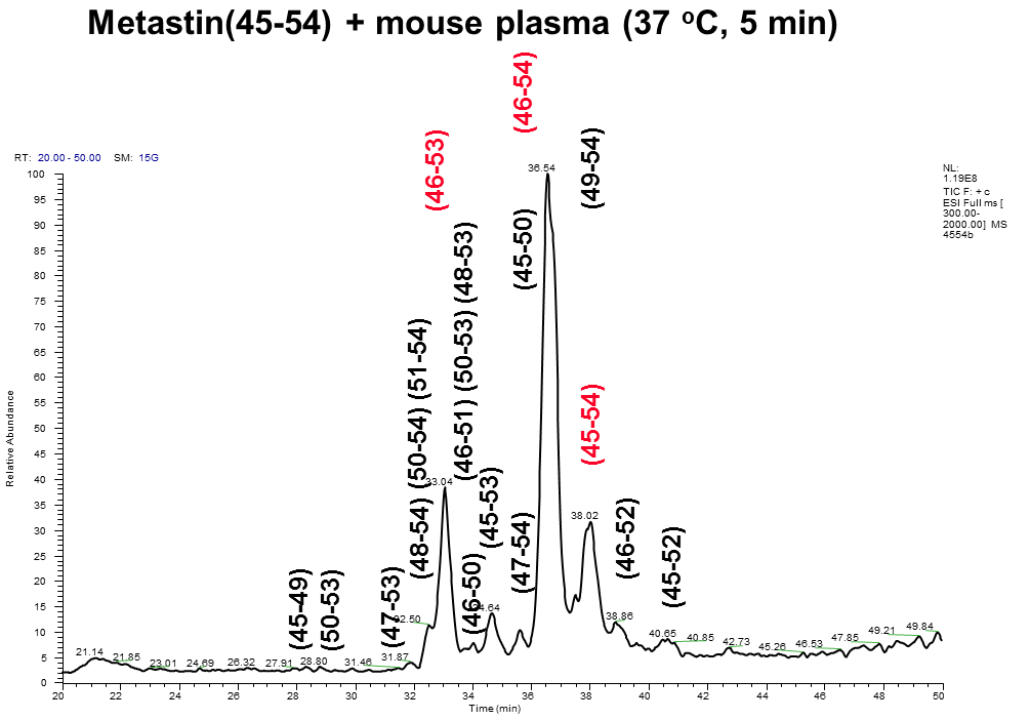


Figure 10. Total ion chromatogram of metastin(45-54) after incubation in mouse plasma.

Arg-containing peptides are known to be good substrates for trypsin-like proteases.[17–19] Therefore, we designed and synthesized a series of short-length KISS1R agonists to examine their agonist activity and trypsin resistance. We used an isolated bovine trypsin to evaluate the stability of these potential agonists against trypsin degradation. In this chapter, we focus on the Arg^{53}-Phe^{54} cleavage and describe a rational and unique strategy for the design of KISS1R agonists resistant to trypsin-like proteases (**Figure 11**).

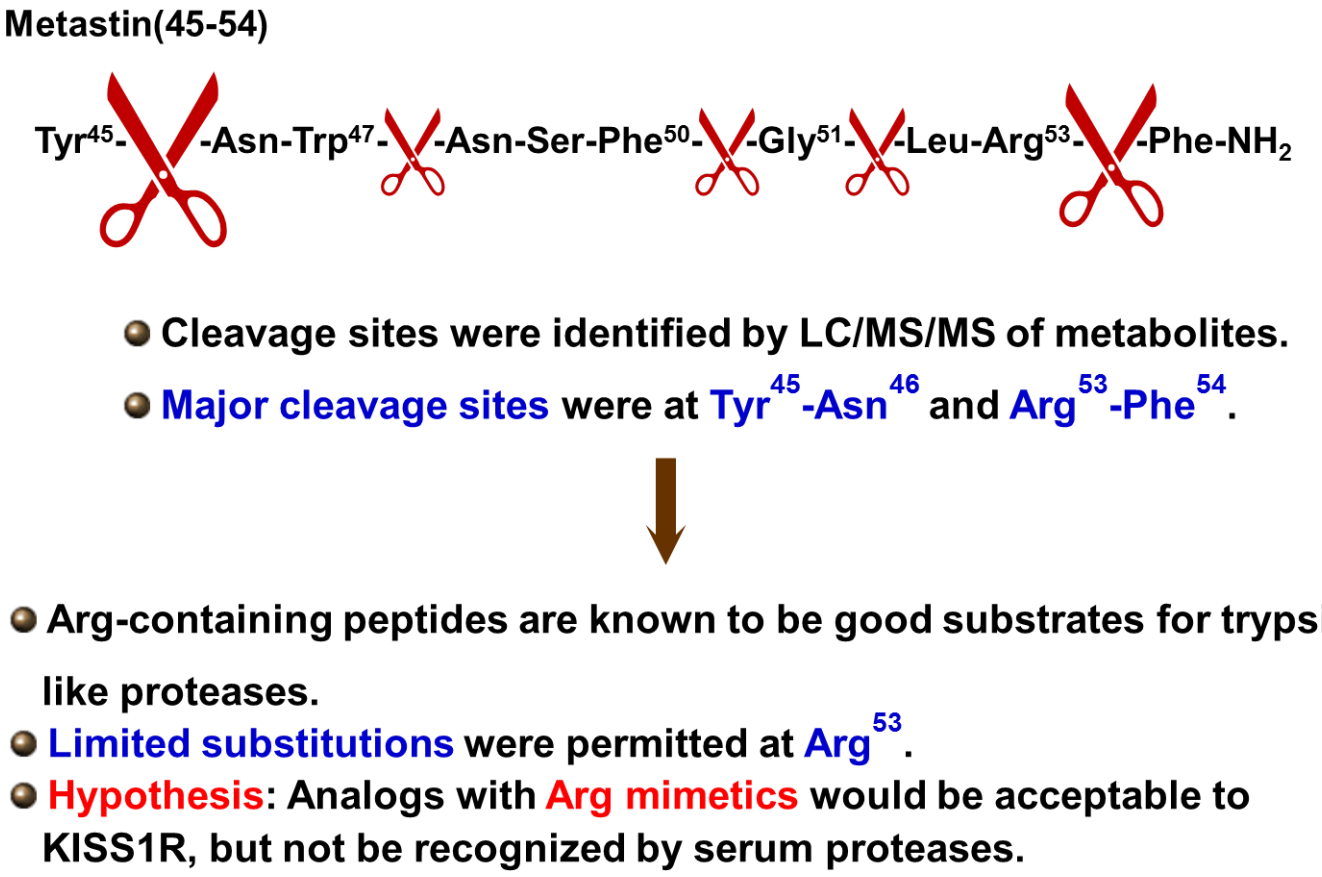


Figure 11. Cleavage sites of metastin(45-54) and peptide design strategy for the improvement of biological stability.

2.4. Design and Synthesis of Metastin(45-54) Analogues Substituted at Position 53.

2.4.1. Chemistry.

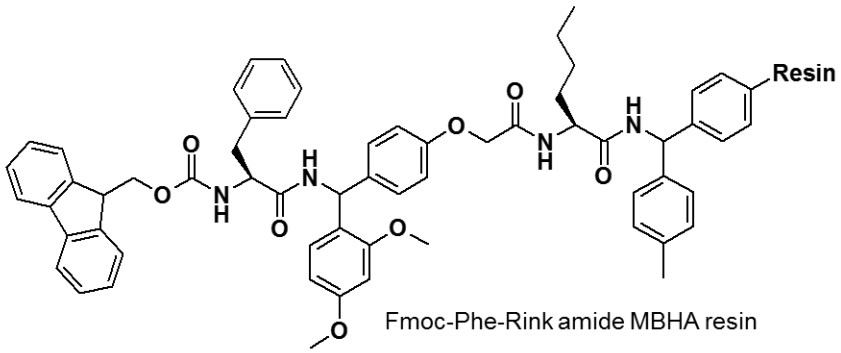
All peptides were synthesized by standard fluorenylmethyloxycarbonyl (Fmoc)-based solid phase synthetic methodology (**Scheme 1**). Crude peptides were purified to homogeneity by preparative HPLC and purity was ascertained by analytical HPLC; matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was performed to assign the structure.

2.4.2. Biological Activities.

The synthesized analogues were characterized by an intracellular calcium ion mobilization assay of KISS1R (hOT7T175) using fluorometric imaging plate reader (FLIPR) technology. The ability to increase intracellular calcium ion levels ($[Ca^{2+}]_i$ activity) of all peptides is shown in **Table 1** as the EC_{50} values (nM) and the specific activity relative to metastin(45-54); higher EC_{50} ratios indicate lower activity compared to metastin(45-54).

2.4.3. Substitution with Neutral Amino Acids.

Metastin contains an RF-amide *C*-terminal structure. The Arg residue in position 53 of metastin(45-54) plays an important role in its biological activity. Metastin analogues in which Arg is substituted with neutral L-amino acids such as alanine (Ala) **1**, leucine (Leu) **2**, norleucine (Nle) **3**, and citrulline (Cit) **4**, which are expected to prevent trypsin recognition and degradation, also showed a 21-112-fold reduction in agonist activities (**Figure 12**, **Table 1**) demonstrating that the presence of a basic amino acid at the position 53 is necessary for the biological activity of metastin(45-54).



→ Boc-Tyr(*t*-Bu)Asn(Trt)Trp(Boc)Asn(Trt)Ser(*t*-Bu)PheGlyLeu-Xaa^{53}Phe-Rink Amide MBHA resin

Stepwise elongation (Fmoc deprotection & coupling)

1) 20% Piperidine/DMF, r.t., 20 min.

2) Fmoc-amino acid (4 eq.), DCC (4 eq.),
HOBt (4 eq.), DMF, r.t., 70 min.

→ TyrAsnTrpAsnSerPheGlyLeu-Xaa^{53}Phe-NH_2

Final deprotection & purification

1) TFA/thioanisole/*m*-cresol/triisopropylsilane/
1,2-ethanedithiol (85/5/5/2.5/2.5), r.t., 30 min.

2) HPLC purification

Scheme 1. General synthetic route to metastin(45-54) analogues

Substitution for Arg[53] with Lys decreased its binding affinity for KISS1R by 10-fold.[20] In our study using the FLIPR assay Lys[53] analogue **5** exhibited 4.2-fold decreased agonist activity compared to metastin(45-54) (**Table 1**).

Table 1. Structures and biological activities of metastin(45-54) analogues substituted at position 53.

H-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-AA[53]-Phe-NH_2

compound	AA[53]	agonist activity[a]	
		EC_{50} (nM)	EC_{50} ratio[b]
metastin(45-54)	Arg	0.065[c]	1.0
1	Ala	1.4	21
2	Leu	1.5	23
3	Nle	5.8	89
4	Cit	7.2	110
5	Lys	0.27	4.2

[a]Agonist activities of all peptide analogues were evaluated in a functional assay of KISS1R (hOT7T175), an intracellular calcium mobilization assay using FLIPR technology.
[b]EC_{50} ratio is the ratio of each peptide EC_{50} value compared with the EC_{50} value of metastin(45-54).
[c]EC_{50} value of metastin(45-54) is calculated as the average value of 14 independent experiments.

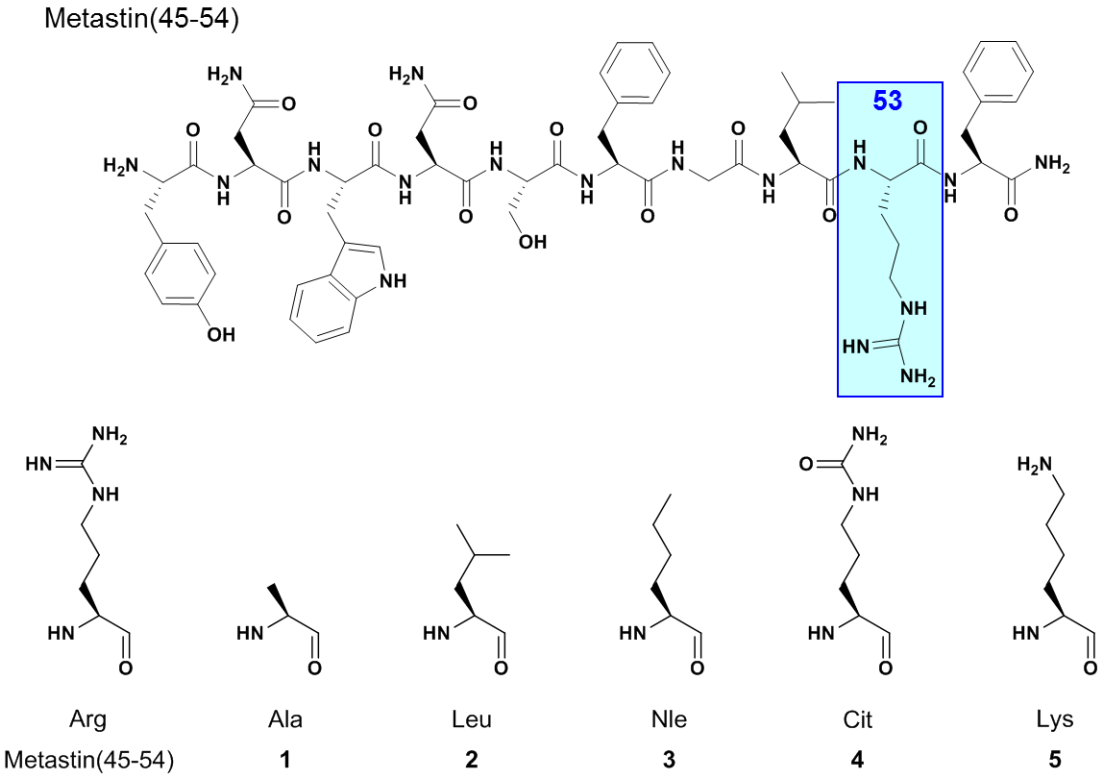


Figure 12. Structures of amino acids at position 53.

2.4.4. Substitution with D-Amino Acid.

It has been widely recognized that substitution of an L-amino acid with the corresponding D-amino acid sometimes provides peptides with improved biological potency because it alters conformational properties of the peptide[21] and increases resistance to enzymatic degradation.[22] However, substitution of Arg[53] with D-Arg in metastin(45-54) markedly decreased its agonist activity for KISS1R[15]. In our studies, the agonist activity of [D-Arg[53]]metastin(45-54) **6** was lower than that of metastin(45-54) by more than three orders of magnitude (**Figure 13**, **Table 2**).

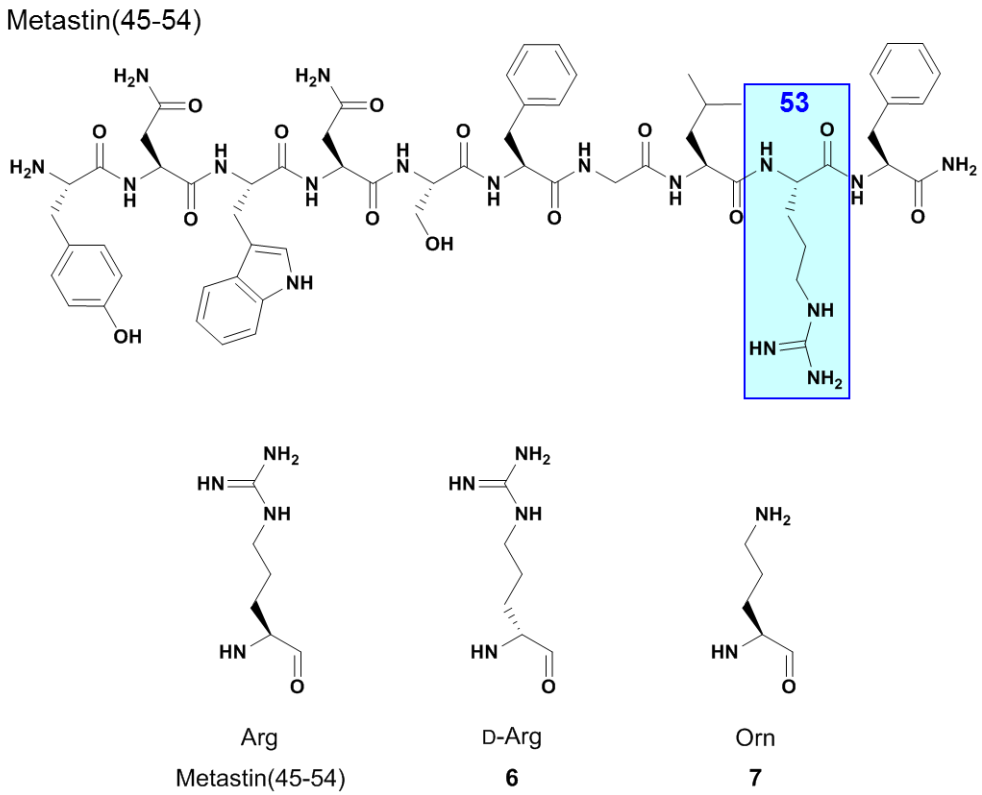


Figure 13. Structures of amino acids at position 53.

Table 2. Structures and biological activities of metastin(45-54) analogues substituted at position 53.

H-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-AA^{53}-Phe-NH_2

compound	AA^{53}	agonist activity[a]	
		EC_{50} (nM)	EC_{50} ratio[b]
metastin(45-54)	Arg	0.065[c]	1.0
6	D-Arg	76	1200
7	Om	0.29	4.5

[a]Agonist activities of all peptide analogues were evaluated in a functional assay of KISS1R (hOT7T175), an intracellular calcium mobilization assay using FLIPR technology.
[b]EC_{50} ratio is the ratio of each peptide EC_{50} value compared with the EC_{50} value of metastin(45-54).
[c]EC_{50} value of metastin(45-54) is calculated as the average value of 14 independent experiments.

2.4.5. Trypsin and Substitution with Ornithine.

Trypsin is known to exhibit a relatively low activity for ornithyl substrates,[23] and it is anticipated that the substitution of Arg would increase the stability of the bond between the residues 53 and 54. However, the replacement of Arg with ornithine (Orn) yielded analogue **7** which had 4.5-fold lower agonist activity (**Figure 13**, **Table 2**).

2.4.6. Substitution with Guanidino-*N*-Alkyl (N^{ω}-Alkyl) Arginine.

Since guanidino-*N*-alkyl (N^{ω}-alkyl) arginine derivatives, which retain the positive charge of Arg, exhibit different lipophilicity and electrostatic characteristics, we originally examined whether the substitution of N^{ω}-alkyl arginine derivatives was effective in the improvement of the *in vivo* agonist potential and serum stability of our analogues. Metastin(45-54) analogues with N^{ω}-alkyl arginine **8**-**12** were synthesized, and their $[Ca^{2+}]_i$-mobilizing activities were compared with that of metastin(45-54) (**Figure 14**).

Because homoarginine (Har) and norarginine (Nar) derivatives have been shown to be resistant to tryptic degradation,[24] we synthesized metastin(45-54) analogues with

substituted Har **13** and Nar **15** and their N^{ω}-methylated derivatives **14** and **16** (**Figure 14**).

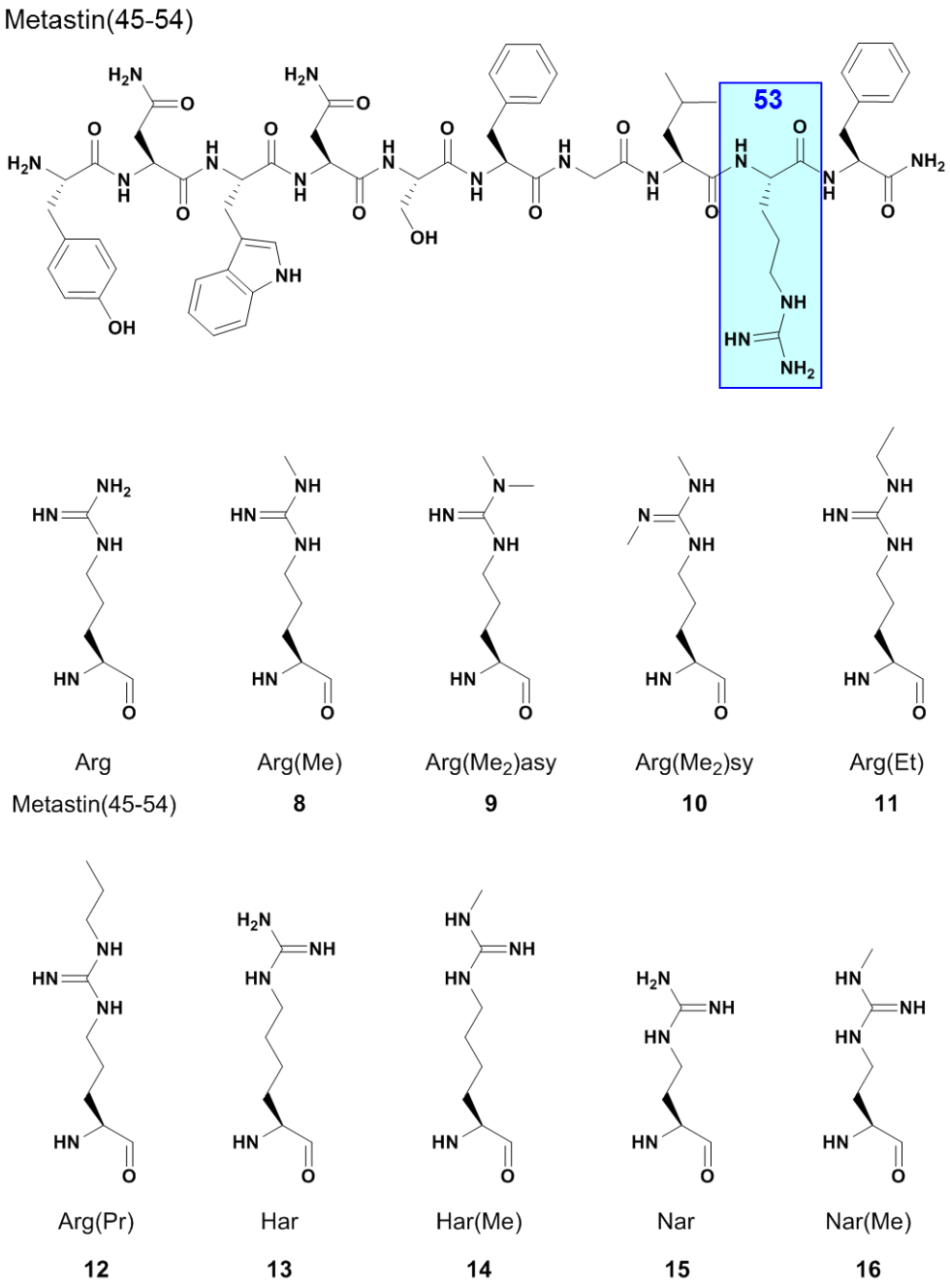


Figure 14. Structures of amino acids at position 53.

Guanylation was performed with 1*H*-pyrazole-1-carboxamidine hydrochloride as the transferable amidine moiety, and methylguanylation was successfully performed using *N*-methyl-*N*,*N*′-bis-Boc-1-guanylpyrazole prepared by methylation of commercially available *N*,*N*′-bis-Boc-1-guanylpyrazole.[25]

In analogues with guanidino moieties, side chain elongation may result in improved receptor interaction. N^{ω}-Methylarginine (Arg(Me)) **8**, $N^{\omega,\omega}$-dimethylarginine (Arg(Me_2)asy) **9**, N^{ω}-ethylarginine (Arg(Et)) **11**, Har **13**, and N^{ω}-methylhomoarginine (Har(Me)) **14** analogues retained their high agonist activities with EC_{50} ratios below 2.1. Analogues **8** and **9** possessed higher $[Ca^{2+}]_i$ mobilization activity compared with metastin(45-54) (**Table 3**).

Table 3. Structures and biological activities of metastin(45-54) analogues substituted at position 53.

H-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-AA^{53}-Phe-NH_2

		agonist activity[a]	
compound	AA^{53}	EC_{50} (nM)	EC_{50} ratio[b]
metastin(45-54)	Arg	0.065[c]	1.0
8	Arg(Me)	0.023	0.36
9	Arg(Me_2)asy	0.039	0.61
10	Arg(Me_2)sym	0.32	4.9
11	Arg(Et)	0.065	1.0
12	Arg(Pr)	0.36	5.6
13	Har	0.029	0.45
14	Har(Me)	0.13	2.1
15	Nar	0.15	2.3
16	Nar(Me)	0.34	5,2

[a]Agonist activities of all peptide analogues were evaluated in a functional assay of KISS1R (hOT7T175), an intracellular calcium mobilization assay using FLIPR technology.
[b]EC_{50} ratio is the ratio of each peptide EC_{50} value compared with the EC_{50} value of metastin(45-54).
[c]EC_{50} value of metastin(45-54) is calculated as the average value of 14 independent experiments.

The N^{ω}-propylarginine (Arg(Pr)) derivative **12**, with a relatively large substituent

group at the guanidino moiety, reduced biological activity by more than 5.6-fold compared to the Arg(Et) derivative **11**. These data suggest that KISS1R can recognize metastin(45-54) with limited modifications at position 53.

2.4.7. Substitution with Other Basic Amino Acids.

In addition to these analogues containing the guanidino moiety at the side chain of position 53, we introduced $N^{\varepsilon,\varepsilon}$-dimethyllysine (Lys($Me_2$)) **17**; 2,4-diaminobutanoic acid (Dbu) **18**; 2,3-diaminopropionic acid (Dpr) **19**; N^{β}-glycyldiaminopropionic acid (Dpr(Gly)) **20**; and N^{β}-(*N*-amidinoglycyl)diaminopropionic acid (Dpr(AmGly)) **21** substitutions at position 53 to investigate the relationship between the structures of the basic side chains and agonist activity (**Figure 15**).

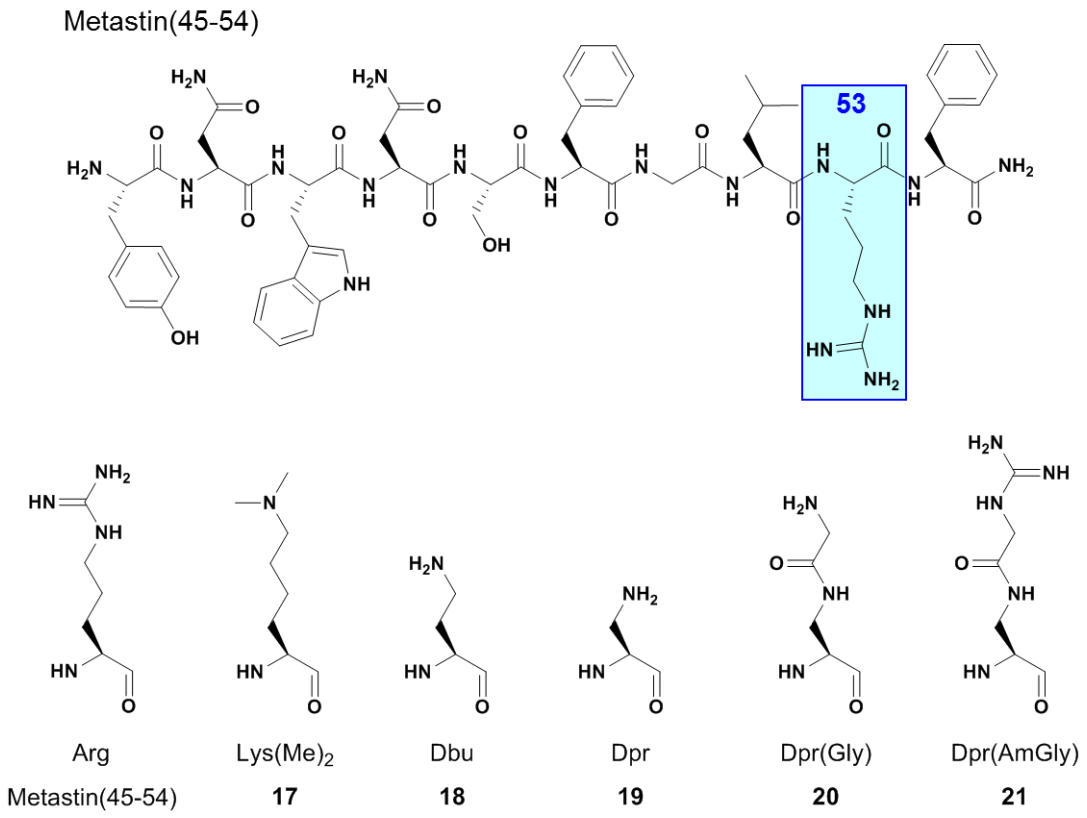


Figure 15. Structures of amino acids at position 53.

Although Lys(Me)$_2$ analogue **17** had the highest agonist activity among the analogues containing unnatural amino acids without guanidino moieties at position 53, its EC_{50} value was 5.1-fold higher than that of metastin(45-54) (**Table 4**). Two analogues with short side chains, **18** and **19**, showed markedly lower agonist activities than Orn analogue **7**. These results indicated that KISS1R recognized not only the structure of the basic moiety but also the distance between the main chain and a particular basic moiety.

Table 4. Structures and biological activities of metastin(45-54) analogues substituted at position 53.

H-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-AA^{53}-Phe-NH_2

		agonist activity[a]	
compound	AA^{53}	EC_{50} (nM)	EC_{50} ratio[b]
metastin(45-54)	Arg	0.065[c]	1.0
17	Lys(Me)$_2$	0.33	5.1
18	Dbu	3.9	61
19	Dpr	1.4	21
20	Dpr(Gly)	10	160
21	Dpr(AmGly)	2.2	34

[a]Agonist activities of all peptide analogues were evaluated in a functional assay of KISS1R (hOT7T175), an intracellular calcium mobilization assay using FLIPR technology.
[b]EC_{50} ratio is the ratio of each peptide EC_{50} value compared with the EC_{50} value of metastin(45-54).
[c]EC_{50} value of metastin(45-54) is calculated as the average value of 14 independent experiments.

2.5. Trypsin Resistance of Metastin(45-54) and its Analogues.

Based on these results, we examined the effect of trypsin degradation on these *N*-terminal truncated human metastin analogues, namely, metastin(45-54), Lys analogue **5,** and Arg(Me) analogue **8.** After 30-min incubation of 0.1 mM peptide with bovine trypsin ((E/S=1/100 (W/W)) in ammonium acetate buffer at pH 8.5, metastin(45-54) and [Lys^{53}]metastin(45-54) **5** were rapidly degraded but Arg(Me) analogue **8** remained intact (**Figure 16**).

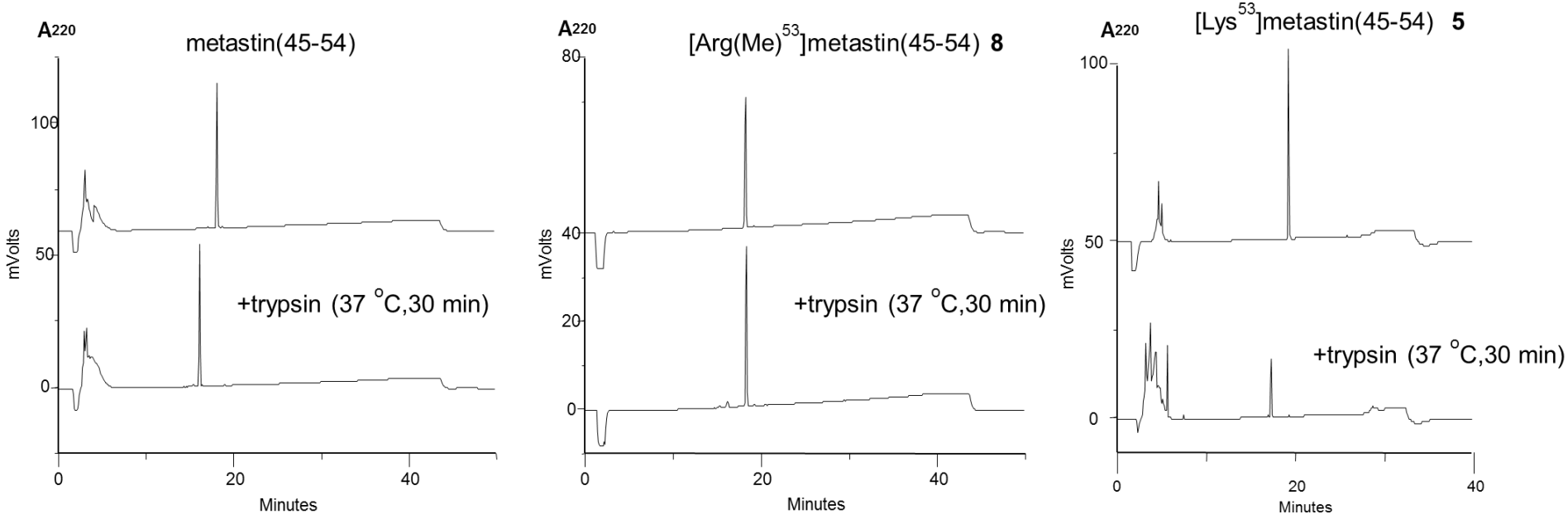


Figure 16. Tryptic digestion of metastin(45-54) and its analogues.

2.6. Serum Stability of Metastin(45-54) and its Analogues.

2.6.1. Stability and Metabolites.

To investigate its metabolic stability in biological fluids, we incubated [Arg(Me)^{53}] metastin(45-54) **8** in 0.1 mM in mouse serum for 5 min. The digest was characterized by LCMS using ion trap mass spectrometry (**Figure 17**). Although enzymatic degradation of analogue **8** in mouse serum yielded several peptide fragments, none were due to cleavage between positions 53 and 54 demonstrating that this Arg(Me) substitution improved serum stability by preventing cleavage between positions 53 and 54. Peak X in the figure was observed as an unknown substance derived from serum protein.

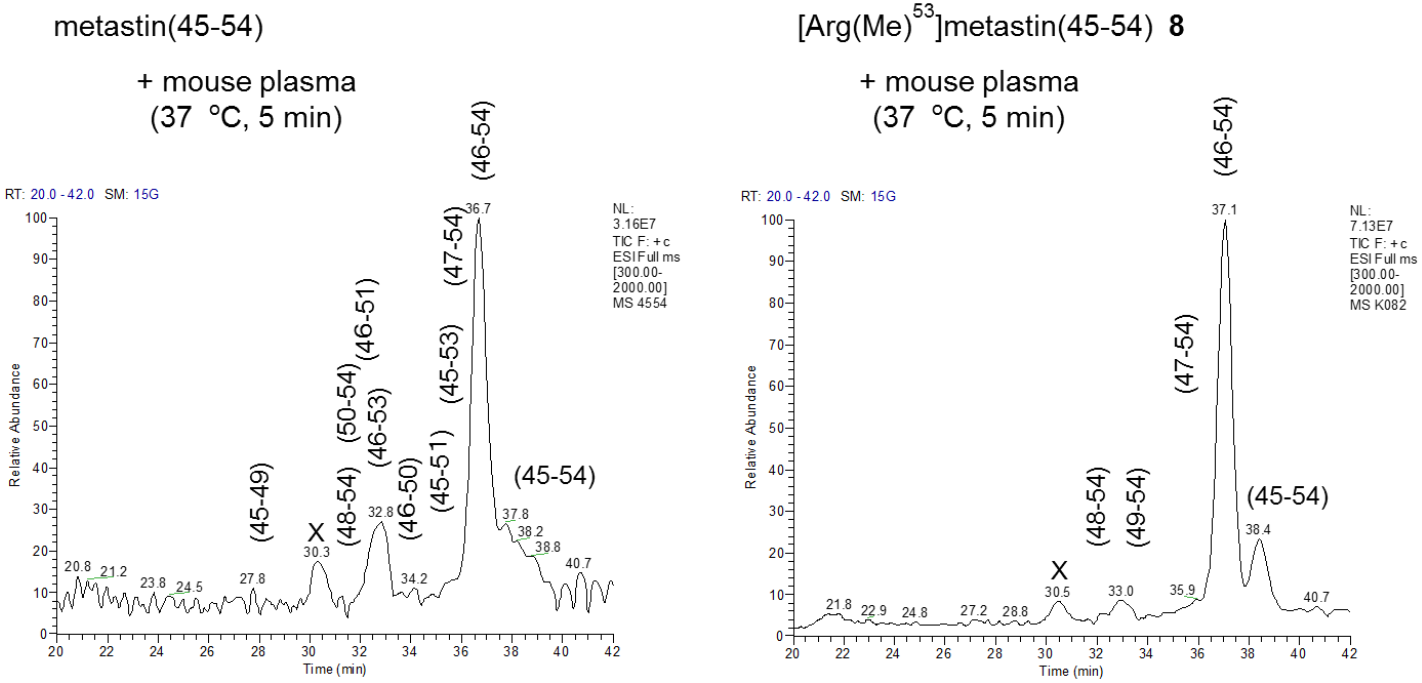


Figure 17. Total ion current chromatogram of metastin(45-54) and [Arg(Me)^{53}]metastin(45-54) **8** in mouse blood samples.

2.6.2. Rate of Degradation.

Kinetic data on the degradation of these compounds in mouse serum are shown in **Figure 18**. Metastin(45-54) or analogue **8** (5 μL of 1 mM aqueous solution) was incubated with 45-μL of mouse serum at 37°C for up to 10 min. At 1, 2, 5, and 10 min, the incubations were stopped by boiling for 3 min. The amount of peptide in serum extracts was determined from the area under HPLC peaks. The percent of a given KISS1R agonist recovered from serum after incubation at 37°C (stability index) was calculated relative to the amount of peptide extracted from serum at time 0.

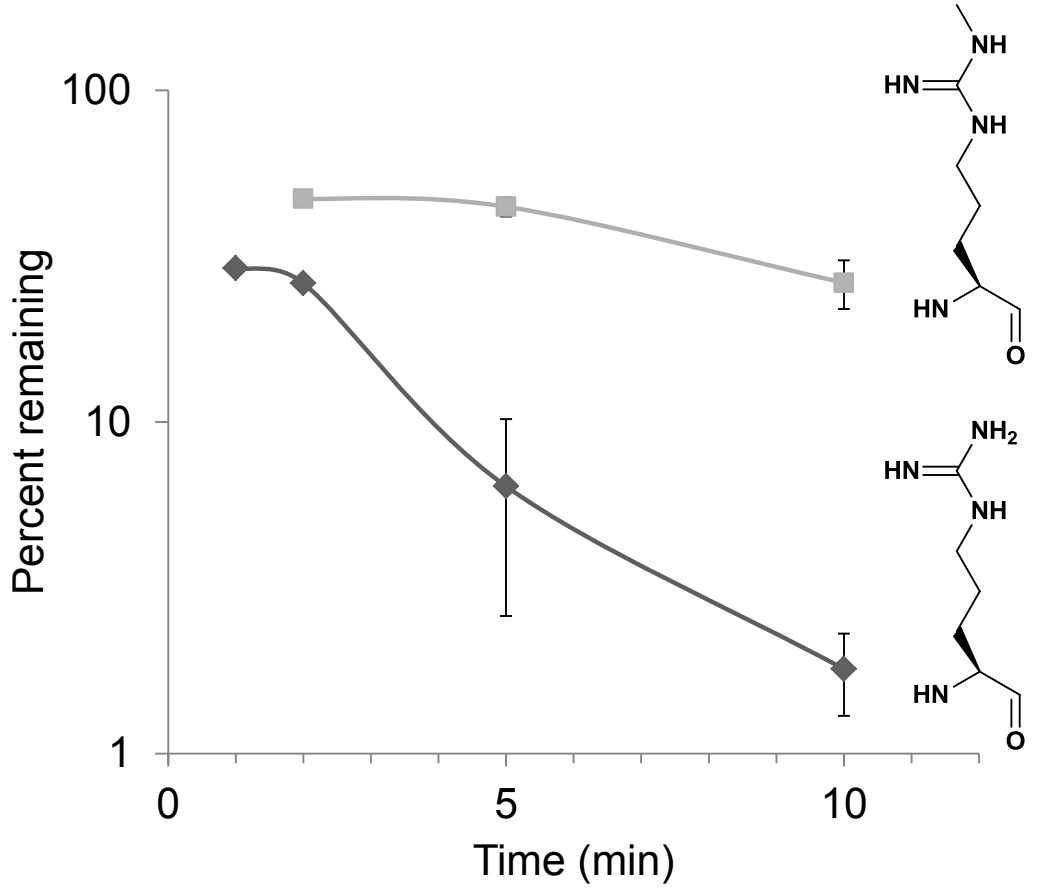


Figure 18. Stability of metastin(45-54) and [Arg(Me)^{53}]metastin(45-54) **8** in mouse blood samples (HPLC analysis).

Rate constants for disappearance and half-life were calculated according to pseudo-first-order kinetic models and optimized using monoexponential regressions. The calculated half-lives of the metastin(45-54) and analogue **8** were 0.56 and 1.8 min, respectively, demonstrating that replacement of Arg by Arg(Me) improved stability of analogue **8** in mouse serum. Other analogues including Har **13**, Har(Me) **14**, and Nar **15** were stable after treatment with trypsin (data not shown).

2.7. Conclusion of Chapter 2

Methylation of the guanidino group of Arg was first recognized more than 40 years ago as a result of post-translational modifications of Arg residues.[26] Our study of metastin(45-54) analogues demonstrated improved peptide stability by substituting Arg residue with Arg(Me). Trypsin-like proteases have been reported to cleave the *C*-terminal end of basic amino acids such as Arg or Lys.[11] However, this is the first report showing that the replacement of Arg by Arg(Me) is a promising approach to prevent the cleavage of an Arg-containing peptide by trypsin-like proteases. In conclusion, the improvement in metabolic stability by Arg(Me) substitution may be useful in the development of other peptide derivatives of metastin(45-54) with higher agonist activity.

Chapter 3 Serum Stability of Selected Decapeptide Agonists of KISS1R Using Pseudopeptides

3.1. Summary of Chapter 3

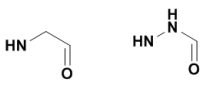
Metastin, a 54-amino acid peptide, is the ligand of the G protein-coupled receptor KISS1R; the *N*-terminally truncated decapeptide, metastin(45-54), has 3–10 times higher receptor affinity and intracellular calcium ion-mobilizing activity but is rapidly inactivated in serum. In this study we designed and synthesized stable KISS1R agonist decapeptide analogues with selected substitutions at positions 47, 50, and 51. Replacement of glycine with azaglycine (azaGly) in which the α-carbon is replaced with a nitrogen atom at position 51 improved the stability of amide bonds between Phe^{50}-Gly^{51} and Gly^{51}-Leu^{52} as determined by *in vitro* mouse serum stability studies. Substitution for tryptophan at position 47 with other amino acids such as serine, threonine, β-(3-pyridyl)alanine, and D-tryptophan (D-Trp), produced analogues that were highly stable in mouse serum. D-Trp^{47} analogue **34** showed not only high metabolic stability but also excellent KISS1R agonist activity. Other labile peptides may have increased serum stability using amino acid substitution.

Metastin(45-54)

Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2

Compound **34**

D-Tyr^{45}-Asn-D-Trp^{47}-Asn-Ser-Phe-azaGly^{51}-Leu-Arg(Me)^{53}-Phe-NH_2

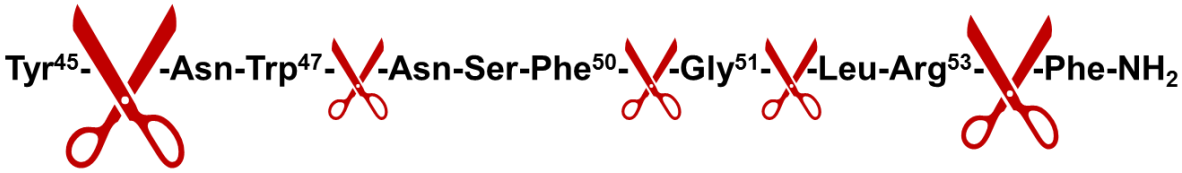


Gly azaGly

3.2. Background and Strategy.

N-Terminally truncated human metastin(45-54), Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2, is 3–10 times more active than metastin *in vitro*.[3] However, both metastin and metastin(45-54) are susceptible to enzyme-catalyzed hydrolysis.[16] As mentioned in the previous chapter, metastin(45-54) was rapidly metabolized in mouse plasma. More than 50% of the peptide was metabolized after a 1-min incubation at 37°C. The metabolites formed during incubation were identified by HPLC-ESI-MS coupling experiments. ESI mass spectra obtained from metastin(45-54) incubated with mouse plasma revealed the presence of more than ten plausible metabolites. Two major peptide fragments, metastin(46-54) and (46-53), were released primarily as a result of Tyr^{45}-Asn^{46} and Arg^{53}-Phe^{54} protease cleavage due to aminopeptidase activity and trypsin-like degradation. We determined that Arg(Me) substitution at the 53 position was an optimal approach to improve serum stability by avoiding cleavage between Arg^{53} and Phe^{54} (**Figure 19**).

Metastin(45-54)



- **Cleavage sites were identified by LC/MS/MS of metabolites.**
- **Major cleavage sites were at Tyr^{45}-Asn^{46} and Arg^{53}-Phe^{54}.**
- **Arg-containing peptides are known to be good substrates for trypsin-like proteases.**
- **$Arg(Me)^{53}$ substitution was found to be acceptable to KISS1R, but not be recognized by serum proteases.**

Compound 8

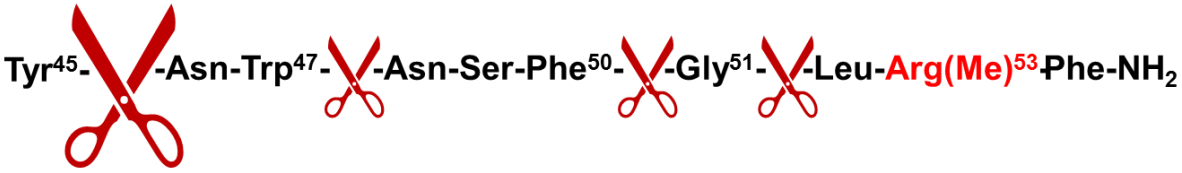


Figure 19. Cleavage sites of metastin(45-54) and compound **8** with improved serum stability.

N^{ω}-Methylarginine [Arg(Me)] substitution has been shown to be highly effective in preventing the cleavage between amino acids Arg[53] and Phe[54]. Consequently, an analogue possessing Arg(Me) was synthesized and found to be metabolically resistant to trypsin-like proteases.

There remains a great demand for more stable analogues with other amino acid replacements at fragile sites to improve their pharmacological properties by maintaining or improving agonist activity and serum stability. In this chapter, a rational strategy is provided for the design of decapeptide analogues of KISS1R agonists to improve their stability in murine serum by focusing on the replacement of cleavage sites. All analogues were designed as ten-amino-acid peptides and evaluated in terms of KISS1R agonist activity, followed by the selection of potent analogues for the further investigation of biological stability using biological fluid.

3.3. Design and Synthesis of Metastin(45-54) Analogues Substituted between Positions 50 and 51.

3.3.1. Substitution with D-Tyrosine (D-Tyr) at Position 45.

Incorporation of D-amino acids has been widely recognized to improve biological potencies of peptides by altering conformational properties[21] and increasing resistance to enzymatic degradation.[22] Peptide **22** with D-Tyr[45] showed acceptable activity and avoided *N*-terminal degradation (**Figure 20**, **Table 5**).

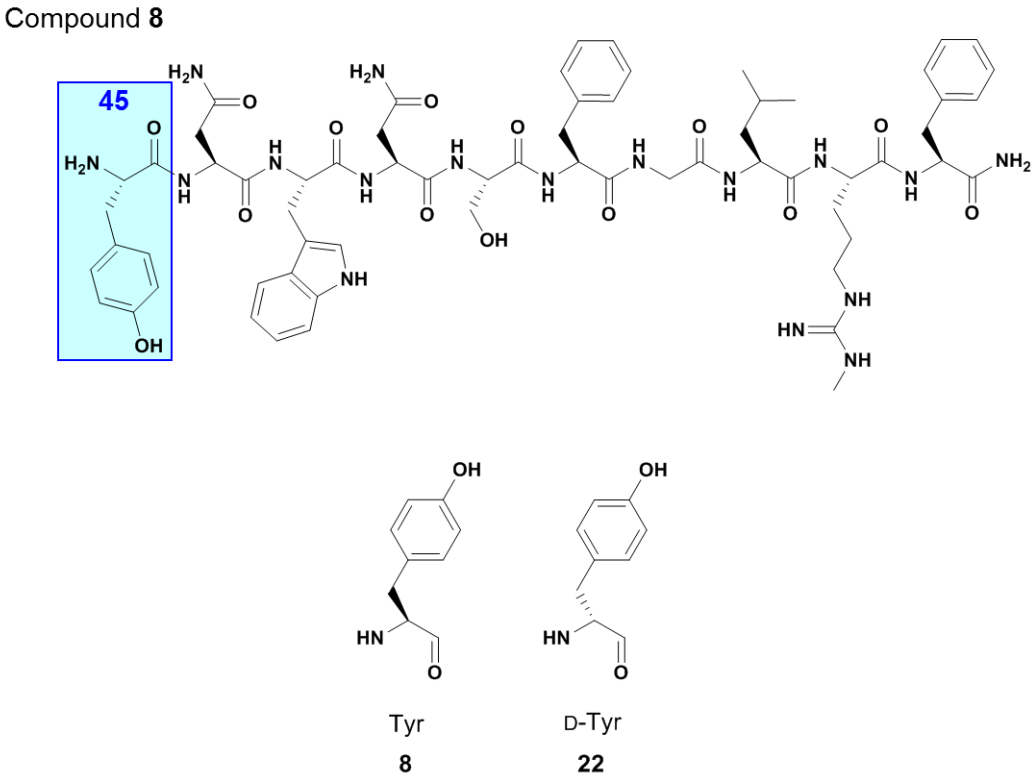


Figure 20. Structures of amino acids at position 45.

Table 5. Biological activities of metastin analogues substituted at position 45.

H-AA[45]-Asn-Trp-Asn-Ser-Phe-Gly-Leu-AA[53]-Phe-NH_2

compound	AA^{45}	AA^{53}	agonist activity[a] EC_{50} (nM)	stability in mouse serum residual ratio (%)[c]
metastin(45-54)	Tyr	Arg	0.049[b]	ND[d]
8	Tyr	Arg(Me)	0.023	ND[d]
22	D-Tyr	Arg(Me)	0.065	18.1

[a]Agonist activities of all peptide analogues were evaluated in a functional assay of KISS1R (hOT7T175), an intracellular calcium mobilization assay using FLIPR technology. EC_{50} values of all peptide analogues were calculated using sigmoidal dose response curves. [b]EC_{50} values of metastin(45-54) were calculated as the average value of 13 independent experiments. [c]Residual ratio after incubation in mouse serum for 1 hr at 37°C. [d]Not determined.

Mouse plasma stability experiments showed that peptide **22** was apparently resistant to proteolysis after a 1 h incubation in mouse serum and showed a treated/control (T/C) ratio of 18.1%, even though several metabolites were observed in the ESI-MS spectra including metastin(45-47), (45-50), (45-51), and (48-54), possibly due to cleavage by chymotrypsin-like proteases, neutral endopeptidases (NEP), and matrix metalloproteinase-9 (MMP-9), as shown in **Figure 21**. Peak X in the figure was observed as an unknown substance derived from serum protein.

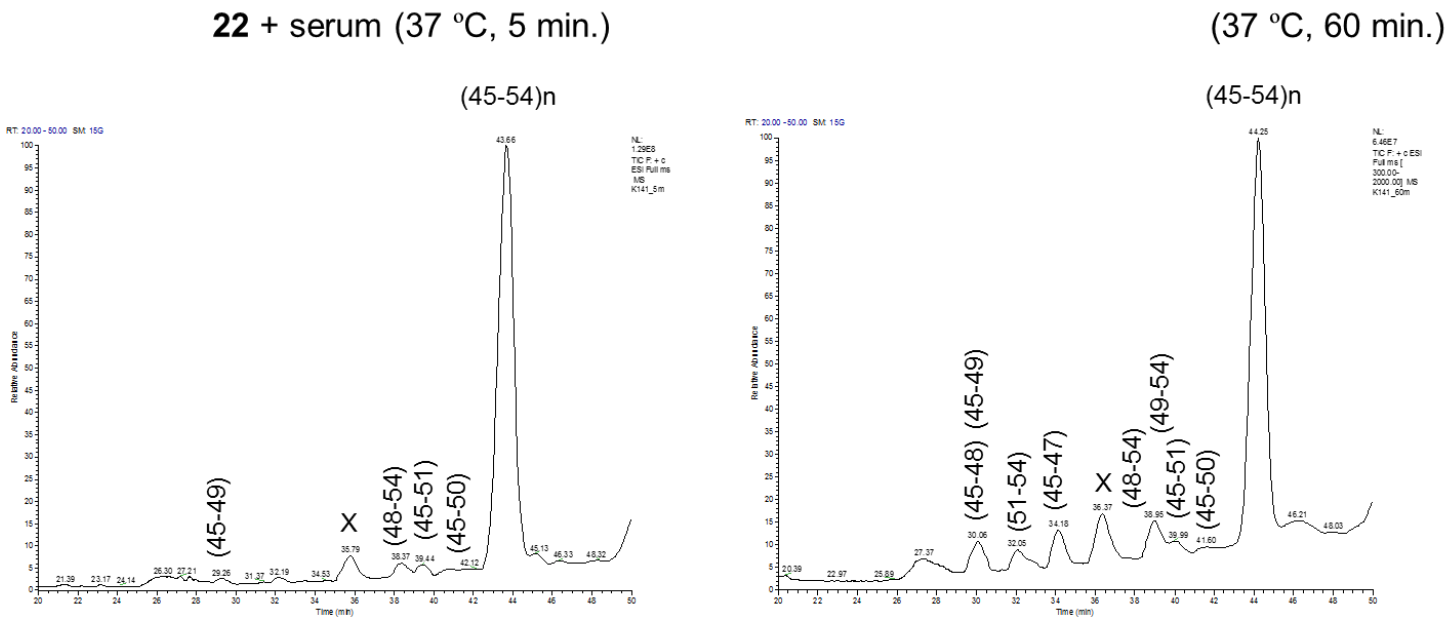


Figure 21. Total ion chromatogram of compound **22** after incubation in mouse serum.

3.3.2. Substitution with D-Amino Acid or *N*-Methyl Amino Acid at Positions 50 and 51.

We hypothesized that more potent and long-acting analogues of compound **22** could be developed if other degradative pathways were successfully inhibited by specific replacement at positions 47, 48, 50, and 51. To examine this hypothesis, we first synthesized a series of analogues with substitution of residues at positions 50 and 51. Preliminary data (not shown) suggested that amino acid substitutions at Phe^{50} and Gly^{51} preserved receptor recognition. D-Amino acid or *N*-methyl amino acid replacement improved metabolic stability but reduced *in vitro* activity.

3.3.3. Substitutions with Dipeptide Isosteres between Positions 50 and 51.

Therefore, we designed and synthesized amide bond isosteres and aza amino acid additions between positions 50 and 51 without altering their side chain moieties (**Figure 22**). This was expected to improve agonist activity and avoid enzymatic degradation by changing the overall conformation of compound **22**.

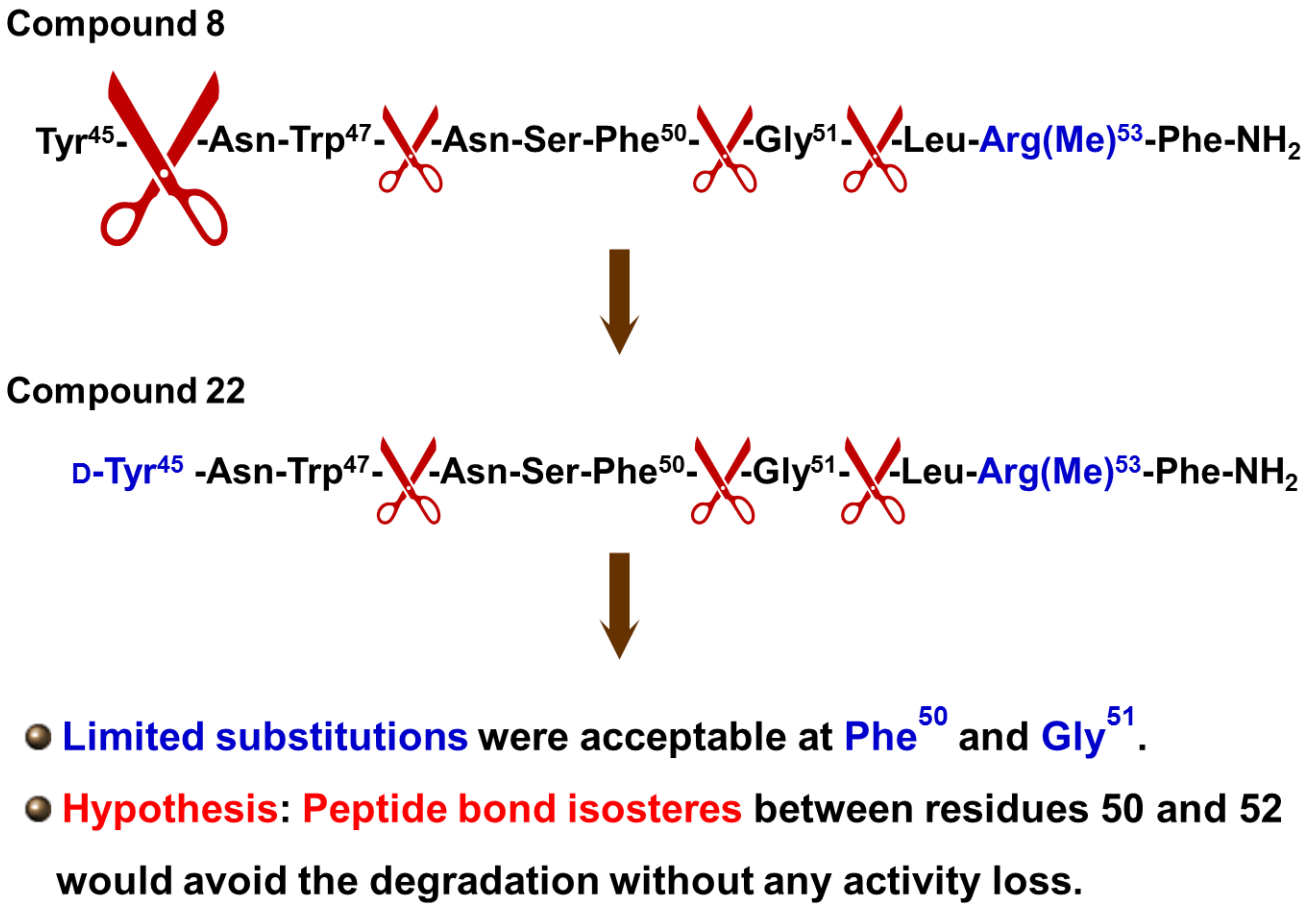


Figure 22. Cleavage sites of compound **22** and peptide design strategy for the improvement of biological stability.

Pseudopeptides have become important for medicinal chemistry.[27] A previous study demonstrated that Gly-Leu dipeptide isosteres, such as (*E*)-alkene- and hydroxyethylene-type isosteres, helped to maintain the agonist activity against MMP-9-mediated cleavage.[27] Metastin analogues with five amino acid residues containing (*E*)-alkene and hydroxyethylene isosteres showed high stability in murine serum.[16]

In this chapter, we designed and synthesized other simple pseudopeptides including thio peptides, reduced amide peptides, retro-inverso peptides and aza peptides (**Figure 23**).

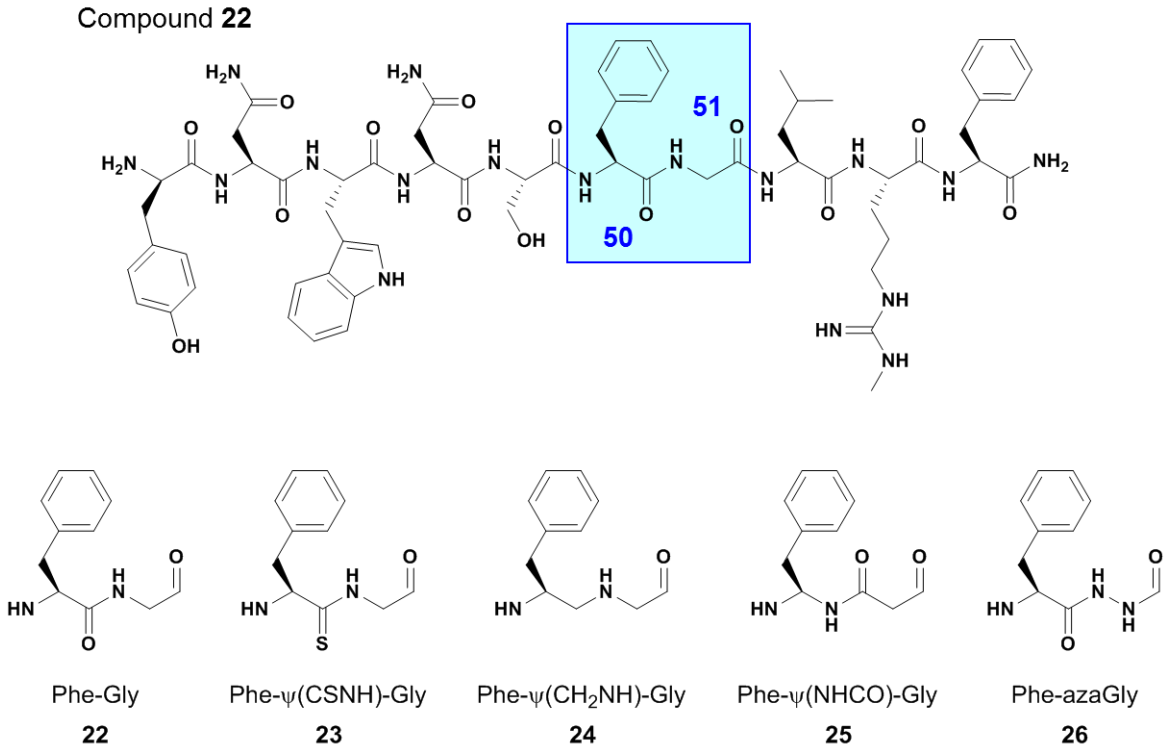


Figure 23. Structures of dipeptide isosteres at positions 50 and 51.

3.3.4. Chemistry, Biological Activities and Serum Stability.

All peptides were synthesized by standard Fmoc-based solid phase synthetic methodology except where specifically described (**Scheme 2**). The $[Ca^{2+}]_i$-mobilizing activities of all peptides are shown as the concentration at 50% of the maximum response (EC_{50} values) calculated using sigmoidal dose response curves in **Tables 6**, **7** and **8**. The receptor binding affinities of the synthesized peptides were determined as IC_{50} values.

The ability of selected peptides to prevent enzymatic hydrolysis was evaluated by incubation in mouse serum at 37°C. The residual ratio, defined as the percent of compound remaining after a 1 h incubation at initial concentrations of 0.1 mM, is given in **Tables 6**, **7** and **8**.

3.3.5. Synthesis Peptides with Dipeptide Isosteres.

Modification of peptides by reduction or thiolation of amide carbonyl oxygen has received attention recently for several reasons. For example, thiolated analogues of biologically active peptides have been reported to show increased enzymatic stability, potency, and selectivity.[28,29] Thiolation of Fmoc-Phe-Gly-O*t*Bu was achieved by means of Lawesson's reagent in toluene at 80 °C for 2 hours, followed by TFA treatment to obtain Fmoc-Phe ϕ (CSNH)Gly-OH.

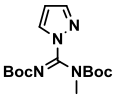
The reduced amide bond (CH_2NH) in compound **24** was formed by reductive alkylation of a preformed Fmoc-Phe-H with H-Leu-Arg(Me,Boc_2)-Phe-Rink amide MBHA resin in the presence of an excess $NaBH_3CN$ and 1% acetic acid in *N*,*N*-dimethylformamide (DMF).[30-32]

Fmoc-Phe-Rink MBHA resin

↓ stepwise elongation (DCC/HOBt)

Fmoc-Orn(Mtt)Phe-Rink MBHA resin

↓ 1) TFA/triisopropylsilane/DCM (1/5/94), r.t., 50 min

↓ 2)  (5 eq.), DIEA (10 eq.), DMF, r.t., 20 h

Fmoc-Arg(Boc_2,Me)Phe-Rink-MBHA resin

↓ 1) 20% piperidine/DMF, r.t., 30 min

↓ 2) Fmoc-Leu, PyBOP, HOBt, DIEA, r.t., 2 h

Fmoc-LeuArg(Boc_2,Me)Phe-Rink-MBHA resin

↓ AA^{50-51}: Phe-Gly — stepwise elongation (DCC/HOBt)

Phe-ψ(CSNH)-Gly
1) 20% piperidine / DMF, r.t., 30 min
2) Fmoc-Pheψ(CSNH)Gly-OH (3 eq.), PyBrop (4 eq.), HOAt (4 eq.), DIEA (8 eq.), r.t., 15 h

Phe-ψ(CH_2NH)-Gly
1) stepwise elongation of Fmoc-Gly (DCC/HOBt)
2) 20% piperidine / DMF, r.t., 30 min
3) Fmoc-Phe-H (5 eq.), $NaBH_3CN$ (5 eq.), AcOH, DMF, r.t., 1 h
4) ZCl (5 eq.), DIEA (5 eq.), DMF, r.t., 3 h

Phe-ψ(NHCO)-Gly
1) 20% piperidine/DMF, r.t., 30 min
2) Fmoc-Pheψ(NHCO)Gly, PyAOP, HOAt, DIEA, DMF, r.t., 6 h
3) Ac_2O, DIEA, DMF, r.t., 30 min

Phe-azaGly
1) 20% piperidine / DMF, r.t., 30 min
2) CDI (4 eq.), THF, r.t., 2 h
3) hydrazine hydrate (8 eq.), r.t., 1 h
4) Fmoc-Phe (4 eq.), PyBrop (4 eq.), HOAt (4 eq.), DIEA (8 eq.), r.t., 2 h

Fmoc-AA^{50-51}-LeuArg(Boc_2,Me)Phe-Rink-MBHA resin

↓ stepwise elongation (DCC/HOBt)

Boc-D-Tyr(*t*-Bu)Asn(Trt)Trp(Boc)Asn(Trt)Ser(*t*-Bu)-AA^{50-51}-LeuArg(Boc_2,Me)Phe-Rink-MBHA resin

↓ AA^{50-51}: Phe-Gly, Phe-ψ(CSNH)-Gly, Phe-ψ(NHCO)-Gly, Phe-azaGly
1) TFA/thioanisole/m-cresol/trisopropylsilane/1,2-ethanedithiol (85/5/5/2.5/2.5), r.t., 2 h
2) HPLC purification

Phe-ψ(CH_2NH)-Gly
1) TMS-Br, thioanisole, *m*-cresol, 1,2-ethanedithiol, TFA, 0 °C, 2 h
2) HPLC purification

↓

D-TyrAsnTrpAsnSer-AA^{50-51}-LeuArg(Me)Phe-NH_2

Scheme 2. Synthetic route to compounds **22**-**26**

Pseudopeptide **25** was synthesized by solid phase synthesis of retro-inverso peptides.[33] The amide functional group of Z-Phe-NH_2 was converted into an amine with bis(trifluoroacetoxy)iodobenzene, followed by condensation with mono-*tert*-butylmalonate and subsequent conversion of the Z group into an Fmoc group. Deprotection of *tert*-butyl ester by TFA treatment for 3 min afforded the desired pseudodipeptide, Fmoc-Phe-ψ(NHCO)-Gly-OH, which was used for the solid phase peptide synthesis.

Aza peptides have an aza amino acid residue in which the α-carbon is replaced with a nitrogen atom. This nitrogen substitution is believed to have little effect on the overall physicochemical profiles of these peptides; however, electronic repulsion between the lone pairs of the adjacent nitrogen atoms of hydrazine moiety has been suggested to induce type I and II β-turn conformations.[34-46]

This β-turn geometry of aza amino acid-containing peptides may contribute to improve binding affinity of metastin analogues for KISS1R. NMR analysis of the metastin(45-54) analogue in dodecylphosphocholine micelles showed that it contained several tight turn structures such as miscellaneous type IV β-turns (residues Asn^{48}–Gly^{51} and Gly^{51}–Phe^{54}).[47] In addition, this manipulation is expected to increase the stability of the aza peptide against proteases that recognize amino acid residues Phe^{50}–Gly^{51} or Gly^{51}–Leu^{52} due to the planar configuration of α-nitrogen between D- and L-amino acids. We designed an aza peptide and synthesized it using solid phase methodology.

3.3.6. Biological Activities of Peptides with Dipeptide Isosteres.

All of these analogues including thio peptide **23**, reduced amide peptide **24**,

retro-inverso peptide **25**, and aza peptide **26**, retained acceptable $[Ca^{2+}]_I$ mobilizing activities and receptor binding activities (**Table 6**). Stability against enzymatic hydrolysis was evaluated in mouse serum and the amount of peptide remaining in serum extracts was determined from the area under HPLC peaks. The stability index defined as the percent of a given metastin analogue recovered from serum after incubation at 37°C for 1 h, was calculated relative to the amount of peptide at time 0 (**Table 6**).

Thio peptide **23**, retro-inverso peptide **25**, and aza peptide **26** displayed increased stability in mouse serum; however, reduced amide peptide **24** exhibited a lower residual ratio compared with peptide **1**. It was assumed that retro-inverso peptide and aza peptide analogues could avoid cleavage of amide bonds between the residues 50 and 51 and 51 and 52. The azaGly analogue was selected for further optimization studies because of its superior water solubility and synthetic characteristics.

Table 6. Biological activities of metastin analogues substituted between positions 50 and 51.

H-AA^{45}-Asn-Trp-Asn-Ser-$AA^{50\text{-}51}$-Leu-AA^{53}-Phe-NH_2

compound	AA^{45}	$AA^{50\text{-}51}$	AA^{53}	agonist activity[a] EC_{50} (nM)	binding affinity[c] IC_{50} (nM) human	rat	stability in mouse serum residual ratio (%)[e]
metastin(45-54)	Tyr	Phe-Gly	Arg	0.049[b]	0.082[d]	0.11[d]	ND[f]
22	D-Tyr	Phe-Gly	Arg(Me)	0.065	0.11	0.26	18.1
23	D-Tyr	Phe-ψ(CSNH)-Gly	Arg(Me)	0.041	2.8	3.3	35.3
24	D-Tyr	Phe-ψ(CH_2NH)-Gly	Arg(Me)	0.62	0.61	1.4	12.1
25	D-Tyr	Phe-ψ(NHCO)-Gly	Arg(Me)	0.95	0.51	2.9	46.8
26	D-Tyr	Phe-azaGly	Arg(Me)	0.050	0.16	0.17	39.5

[a]Agonist activities of all peptide analogues were evaluated in a functional assay of KISS1R (hOT7T175), an intracellular calcium mobilization assay using FLIPR technology. EC_{50} values of all peptide analogues were calculated using sigmoidal dose response curves. [b]EC_{50} values of metastin(45-54) were calculated as the average value of 13 independent experiments. [c]Receptor binding affinities of the synthesized peptides were determined as IC_{50} values. [d]IC_{50} value of metastin(45-54) was calculated as the average value of three independent experiments. [e]Residual ratio after incubation in mouse serum for 1 hr at 37°C. [f]Not determined.

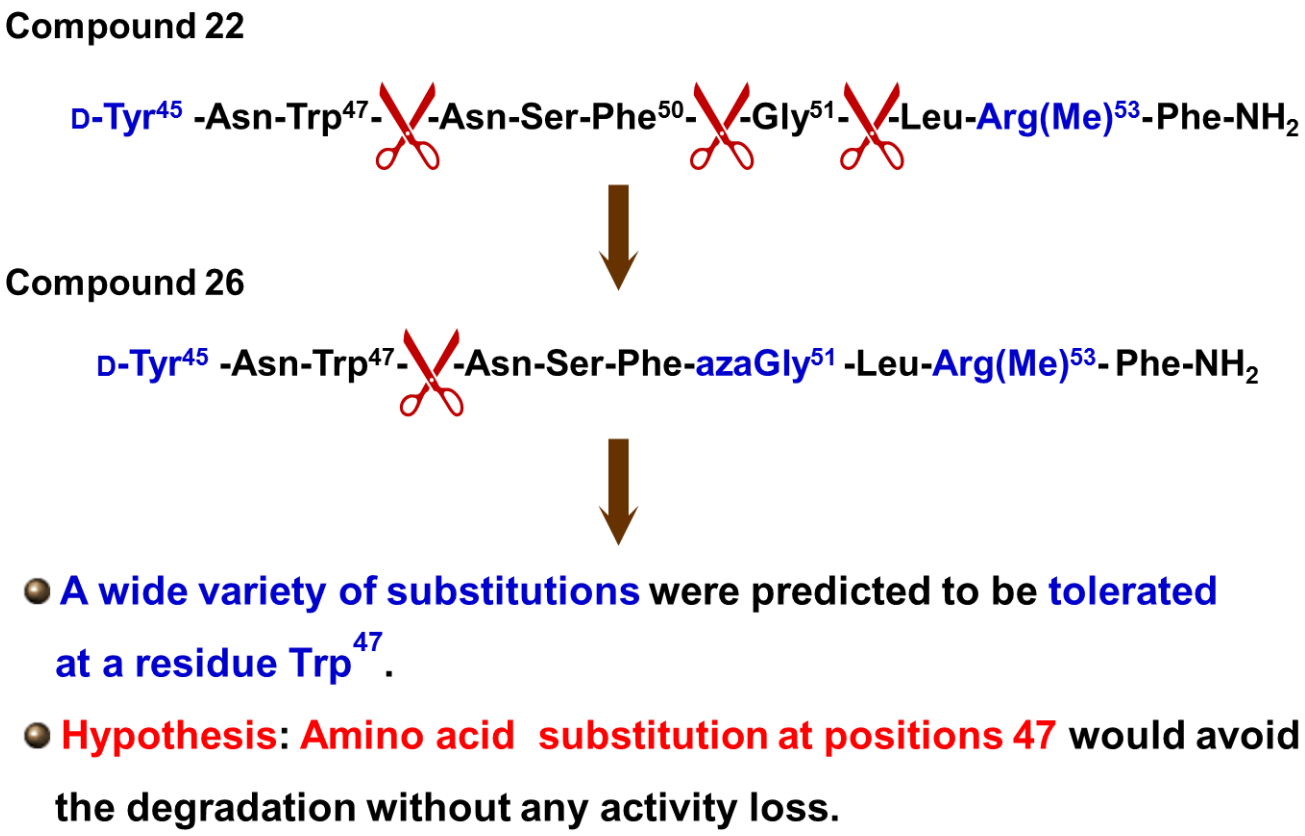


Figure 24. Cleavage sites of compound **26** and peptide design strategy for the improvement of biological stability.

3.4. Design and Synthesis of Metastin(45-54) Analogues Substituted at Position 47.

3.4.1. Substitution with Unnatural and Hydrophilic Amino Acids at Position 47.

The sequence containing tryptophan at position 47 was prone to enzymatic degradation by chymotrypsin-like proteases which are known to exhibit high affinity for aromatic amino acid substrates such as phenylalanine, tyrosine, and tryptophan.[48,49] On the other hand, it was expected that these chymotrypsin-like proteases would exert a relatively low activity against substrates that had unnatural or hydrophilic amino acids.

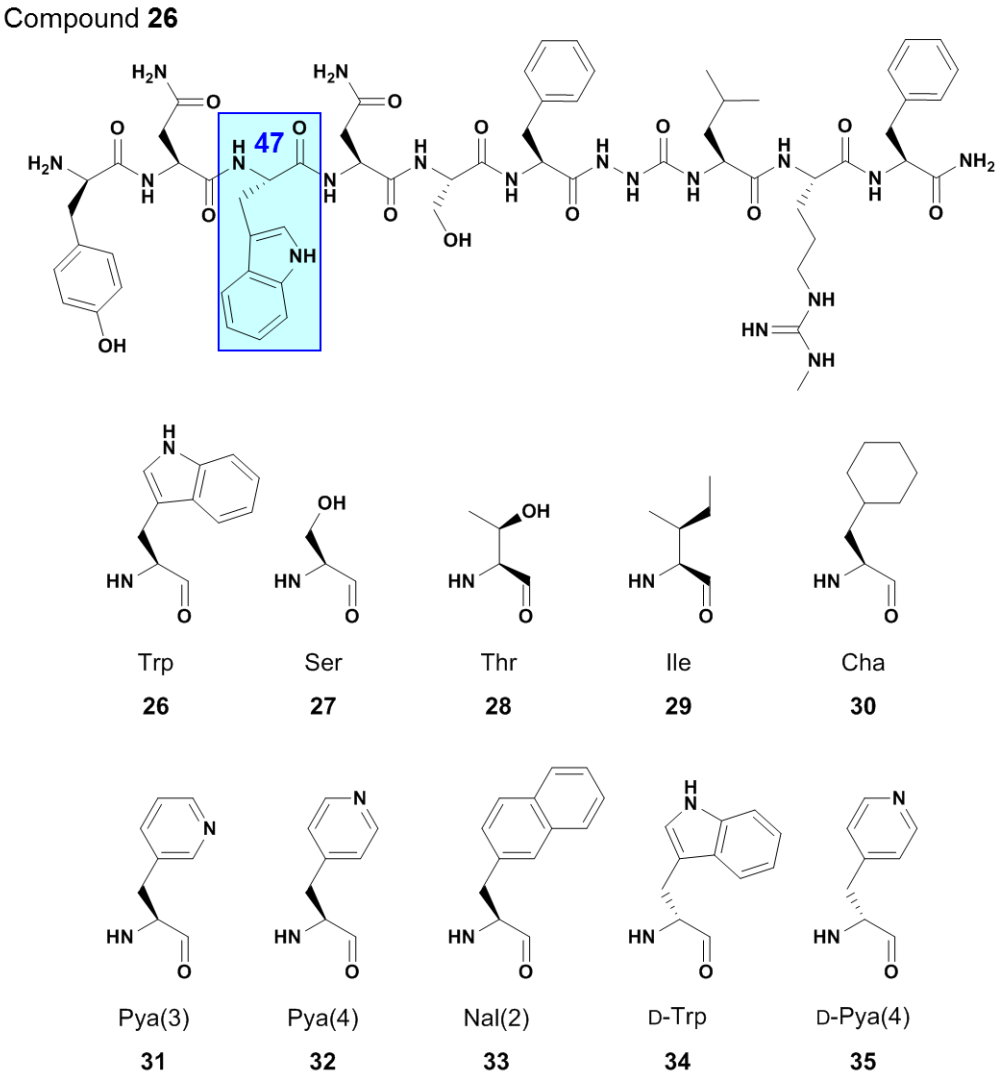


Figure 25. Structures of amino acids at position 47.

Based on this, the following modifications were carried out to increase the stability of the bond between residues 47 and 48 (**Figure 24**, **25**).

3.4.2. Substitution with Serine at Position 47.

Replacement of Trp^{47} with serine (Ser) yielded analogue **27** with relatively low bioactivity but superior metabolic stability compared with compound **26** (**Table 7**). The residual ratio of compound **27** after a 1 h incubation was 50.0%.

Table 7. Biological activities of decapeptide metastin analogues substituted at position 47.

H-D-Tyr-Asn-AA^{47}-Asn-Ser-Phe-azaGly-Leu-Arg(Me)-Phe-NH_2

		agonist activity[a]	binding affinity[b] IC_{50} (nM)		stability in mouse serum
compound	AA^{47}	EC_{50} (nM)	human	rat	residual ratio (%)[c]
26	Trp	0.050	0.16	0.17	39.5
27	Ser	0.73	0.49	0.43	50.0

[a]Agonist activities of all peptide analogues were evaluated in a functional assay of KISS1R (hOT7T175), an intracellular calcium mobilization assay using FLIPR technology. EC_{50} values of all peptide analogues were calculated using sigmoidal dose response curves. [b]Receptor binding affinities of the synthesized peptides were determined as IC_{50} values. [c]Residual ratio after incubation in mouse serum for 1 hr at 37°C.

Several metabolites were identified by HPLC-ESI-MS coupling experiments following a 1 h incubation of compound **27** which was a prototype model peptide with an azaGly residue (**Figure 26**). Peak X in the figure was also observed as an unknown substance derived from serum protein. Compound **27** was not cleaved between positions 50 and 52, and resisted proteolytic degradation in a 15 min human serum incubation in which metastin(45-54) showed rapid decomposition (**Table 8**).

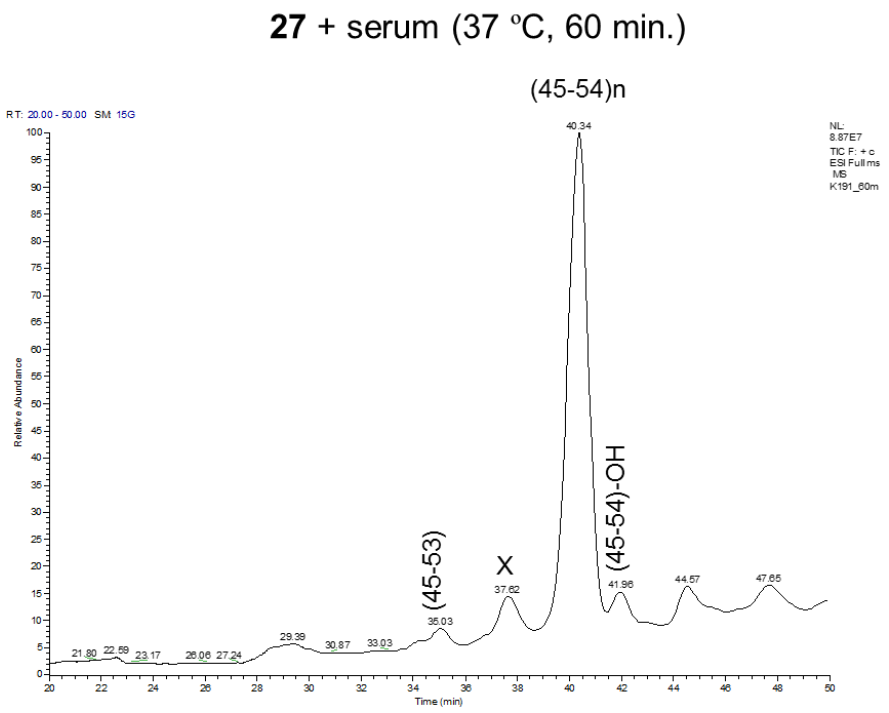


Figure 26. Total ion chromatogram of compound **27** after incubation in mouse serum.

Table 8. Stabilities of metastin analogues in human serum.

compound	residual ratio[a]				$t_{1/2}$[b] (min)
	0 min	5 min	10 min	15 min	
metastin(45-54)	66	0.9	<0.5	<0.5	<1
22	93.4	90.7	85.8	82.7	75
27	86.5	86.5	85.3	86.4	stable

[a]Residual ratio was determined after incubation in human serum 1 hr at 37°C (n=2). [b]The half-lives of studied analogues were calculated according to pseudo-first-order kinetic models and optimized using monoexponential regressions.

3.4.3. Substitution with a Variety of Amino Acids at Position 47.

KISS1R recognition has been reported to be relatively unaffected by the structure of the *N*-terminal region of metastin(45-54). D-Amino acid substitutions at positions 45, 46, and 47 in metastin(45-54) retained potent biological activity comparable to metastin(45-54),[50,51] suggesting that optimization at residue 47 with L- or D-amino acids

would result in novel analogues with both high $[Ca^{2+}]_i$ mobilizing activities and serum stability.

AzaGly[51] derivatives substituted with unnatural amino acids for Trp[47], β-cyclohexylalanine (Cha) **30**, β-(4-pyridyl)alanine [Pya(4)] **32**, and β-(2-naphthyl)alanine [Nal(2)] **33** analogues demonstrated comparable $[Ca^{2+}]_i$-mobilizing activities compared with Trp analogue **26** (**Table 9**). Of these analogues, Nal(2) analogue **33** showed the highest activity and was nearly equivalent to that of metastin(45-54). However, the residual ratios of these compounds was less than 50%.

Table 9. Biological activities of decapeptide metastin analogues substituted at position 47.

H-D-Tyr-Asn-AA[47]-Asn-Ser-Phe-azaGly-Leu-Arg(Me)-Phe-NH_2

		agonist activity[a]	binding affinity[b] IC_{50} (nM)		stability in mouse serum
compound	AA^{47}	EC_{50} (nM)	human	rat	residual ratio (%)[c]
26	Trp	0.050	0.16	0.17	39.5
27	Ser	0.73	0.49	0.43	50.0
28	Thr	0.24	0.86	0.83	62.5
29	Ile	0.13	0.43	0.46	ND[d]
30	Cha	0.10	0.19	0.24	32.0
31	Pya(3)	0.24	0.70	0.89	50.6
32	Pya(4)	0.11	0.30	0.28	30.7
33	Nal(2)	0.098	0.13	0.20	43.6
34	D-Trp	0.072	0.19	0.22	56.4
35	D-Pya(4)	0.59	0.28	0.28	ND[d]

[a]Agonist activities of all peptide analogues were evaluated in a functional assay KISS1R (hOT7T175), an intracellular calcium mobilization assay using FLIPR technology. EC_{50} values of all peptide analogues were calculated using sigmoidal dose response curves. [b]Receptor binding affinities of the synthesized peptides were determined as IC_{50} values. [c]Residual ratio after incubation in mouse serum for 1 hr at 37°C. [d]Not determined.

Other L-amino acid substitutions such as threonine (Thr) **28**, isoleucine (Ile) **29**, and β-(3-pyridyl)alanine [Pya(3)] **31**, showed decreased agonist activities compared to metastin(45-54), although the residual ratios of peptides **28** and **31** were above 50% in serum stability studies.

As for D-amino acid substitutions, D-Trp^{47} **34** and D-Pya(4)^{47} **35** analogues exhibited good receptor binding affinity, which were comparable to the corresponding L-form amino acid derivatives **26** and **32**. This result suggested that the stereochemistry of the α-carbon at position 47 does not play a critical role in KISS1R recognition, as expected. In addition, D-Trp analogue **34** showed excellent serum stability with a residual ratio of 56.4%, compared to 39.5% for Trp analogue **26**.

3.5. Conclusion of Chapter 3.

In summary, we designed and synthesized metabolically stable KISS1R decapeptides with selected substitutions at positions 47, 50, and 51. Replacement of Gly with an azaGly residue, which involved a simple nitrogen atom replacement of an α-carbon at Gly, resulted in good agonist activity for KISS1R. This indicated that some β-turn conformations, which are reported to be observed in aza peptides, may contribute to interactions with KISS1R. Additionally, incorporation of the azaGly^{51} residue improved metabolic stability of the Phe^{50}-Gly^{51} and Gly^{51}-Leu^{52} bonds, which were resistant to serum proteases such as chymotrypsin, NEP, and MMP-9. Although a variety of aza amino acid analogues of biologically active peptides has been reported with improved biological potency,[52-63] this study is the first report that aza amino acid replacement is a simple way to protect both fragile amide bonds at the *N*-terminal and *C*-terminal of the aza amino acid residue.

Substitution of Trp at position 47 with other amino acids, exemplified in this series of analogues with azaGly^{51}, produced several analogues that were stable to metabolic degradation. Our study demonstrated that the key amino acid replacement with azaGly^{51} resulted in metabolically stable analogues that could be easily synthesized using solid-phase methodology without preparing dipeptide isostere units. Further optimization studies on novel azaGly short length metastin agonists with high potency and metabolic stability are described in the next chapter.

Chapter 4 Design, Synthesis, and Biological Evaluation of Novel Investigational Nonapeptide KISS1R Agonists with Testosterone-suppressive Activity

4.1. Summary of Chapter 4.

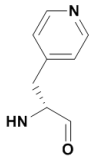
Metastin is a 54 amino acid peptide ligand of the KISS1R receptor and is a critical regulator of GnRH secretion. The *N*-terminally truncated peptide, metastin(45-54), possesses 10-fold higher receptor-binding affinity and agonist KISS1R activity but is rapidly inactivated in rodent plasma. We have developed a decapeptide analogue [D-Tyr^{45},D-Trp^{47},azaGly^{51},Arg(Me)^{53}]metastin(45-54) **34**, with improved serum stability compared with metastin(45-54), but decreased KISS1R agonist activity. Amino acid replacements at positions 45-47 led to enhancement of KISS1R agonist activity and metabolic stability. *N*-terminal truncation resulted in a stable nonapeptide, [D-Tyr^{46},D-Pya(4)^{47},azaGly^{51},Arg(Me)^{53}]metastin(46-54), compound **56,** which displayed KISS1R binding affinities comparable to metastin(45-54) and had improved serum stability.

Metastin(45-54)

Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2

Compound **56**

D-Tyr^{46}-D-Pya(4)^{47}-Asn-Ser-Phe-azaGly^{51}-Leu-Arg(Me)^{53}-Phe-NH_2



D-Pya(4)

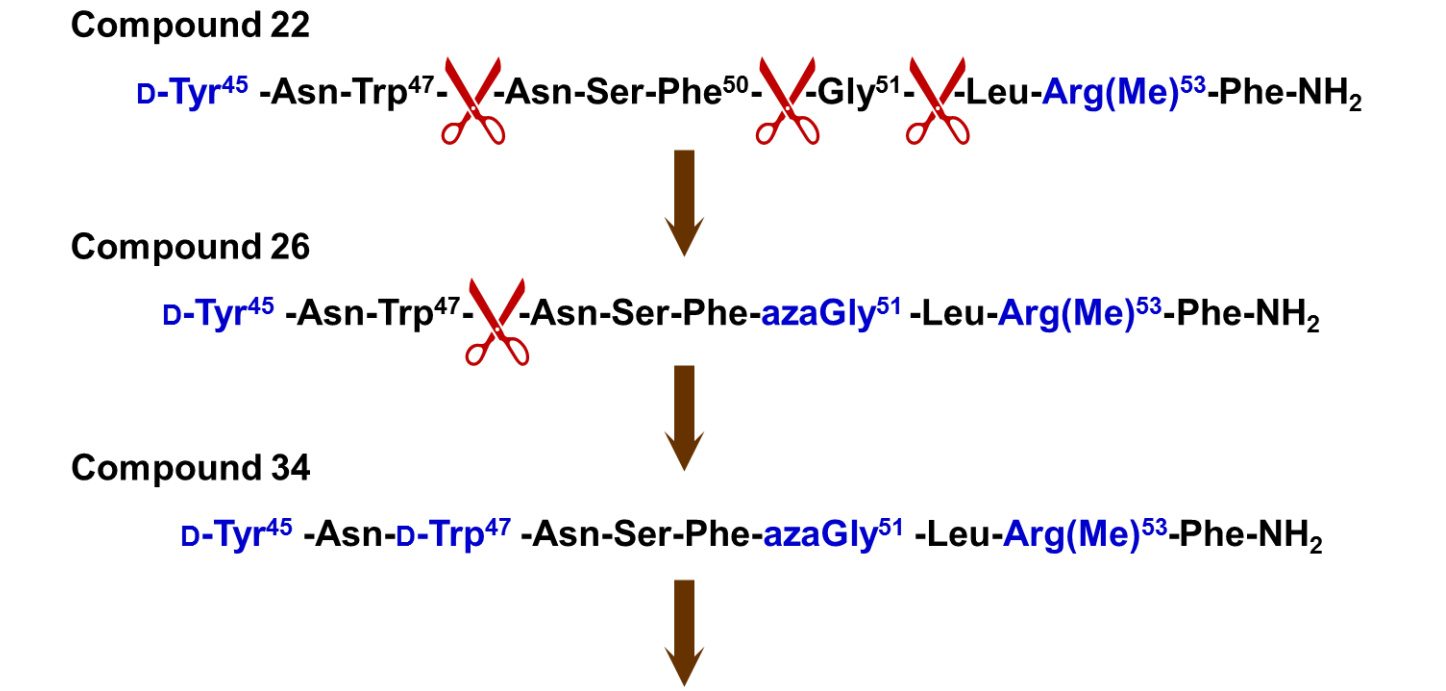
Compound **56** reduced plasma testosterone in male rats and is the first short-length metastin analogue to possess testosterone suppressive activities. Compound **56** has led to the elucidation of investigational analogues TAK-683 and TAK-448, both of which have undergone clinical evaluation for hormone-dependent diseases such as prostate cancer.

4.2. Background and Strategy.

Native metastin contains 54 peptide residues, but an *N*-terminally truncated analogue metastin(45-54) displays full biological activities *in vitro*. This fragment peptide is three- to ten-fold more active than native metastin,[3] indicating that the active core of biological activity resides in the *C*-terminal region of metastin.

Peptides are often not regarded as promising drug candidates because peptide bonds can be enzymatically fragile frequently resulting in rapid degradation by various serum proteases. Modifications of peptides can sometimes increase metabolic stability and improve their potential therapeutic utility.

In the previous chapters, rational modifications of metastin(45-54) lead to the development of [D-Tyr^{45},Arg(Me)^{53}]metastin(45-54) **22**, which had greater metabolic stability than metastin(45-54). Both D-tyrosine (D-Tyr) at position 45 and N^{ω}-methylarginine [Arg(Me)] at position 53 substitutions were acceptable and maintained KISS1R activity while avoiding *N*-terminal and *C*-terminal degradations, respectively. Metabolic stability was further improved by azaGly substitution at position 51. Replacement of Gly by an azaGly residue improved stability for the 50-51 and 51-52 bonds, which was expected to confer resistance to serum proteases such as chymotrypsin, NEP, and MMP-9. Substitution of Trp in position 47 with Ser or D-Trp also improved metabolic stability.



- **A wide variety of substitutions were predicted to be tolerated between residues D-Tyr^{45} and D-Trp^{47}.**
- **Hypothesis: Combination of amino acid substitutions/deletions between positions 45 and 47 would improve biological activity and stability.**

Figure 27. Cleavage sites of compound **22** and **26**; peptide design strategy for the improvement of biological activity and stability.

Building on these earlier investigations, the aim of the present study was to create metastin analogues with improved resistance to serum proteases while maintaining a high metastin agonist activity compared with our lead compound **34** (**Figure 27**). All analogues were designed as decapeptides or nonapeptides, and evaluated in terms of KISS1R agonist activity and binding affinity, followed by the selection of potent analogues for the further investigation of biological stability using biological fluid. Moreover, biological investigation *in vitro* and *in vivo* of selected compounds was planned for the elucidation of roles of metastin and KISS1R.

4.3. Design and Synthesis of KISS1R Agonists Substituted between Positions 45 and 47.

4.3.1. Substitution with Pya(4), D-Trp, D-Pya(4) at Position 47.

Based on the study described in the previous chapter of decapeptide metastin(45-54) analogues, azaGly^{51} was shown to be a key structure for good agonist activity and enzymatic stability in mouse serum. Analogues with an azaGly^{51} moiety avoided cleavage by proteases at positions 50-51 and 51-52. In further optimization studies performed here, azaGly^{51} derivatives which substituted for Trp^{47} with D-Trp^{47} **34** and D-Pya(4)^{47} **35**, which were expected to avoid enzymatic degradation, had calcium mobilization activity comparable to corresponding L-amino acid derivatives **26** and **32** (**Table 10**). These results implied that the stereochemical configuration of the side chain at position 47 did not play a critical role in receptor recognition. A wide variety of substitutions were predicted to be tolerated between residues D-Tyr^{45} and Trp^{47}. Combination of amino acid substitutions/deletions between positions 45 and 47 was thought to show improved biological activity and stability of metastin analogues (**Figure 27**).

Table 10. Biological activity of decapeptide metastin analogues.

H-AA^{45}-Asn-AA^{47}-Asn-Ser-Phe-AA^{51}-Leu-AA^{53}-Phe-NH_2

					agonist activity[a] EC_{50} ratio	affinities Ki nM (95% CI)[b]		stability in mouse serum
compound	AA^{45}	AA^{47}	AA^{51}	AA^{53}	human	human	rat	residual ratio (%)[c]
metastin(45-54)	Tyr	Trp	Gly	Arg	1.0	0.039 (0.032-0.047)	0.057 (0.035-0.093)	ND[d]
22	D-Tyr	Trp	Gly	Arg(Me)	0.93	0.046 (0.026-0.081)	0.11 (0.069-0.18)	18.1
36	Tyr	Trp	azaGly	Arg	1.7	0.045 (0.037-0.054)	0.040 (0.030-0.055)	ND[d]
26	D-Tyr	Trp	azaGly	Arg(Me)	2.8	0.051 (0.040-0.064)	0.068 (0.048-0.096)	39.5
32	D-Tyr	Pya(4)	azaGly	Arg(Me)	5.0	0.12 (0.068-0.22)	0.12 (0.066-0.20)	30.7
34	D-Tyr	D-Trp	azaGly	Arg(Me)	3.7	0.078 (0.064-0.094)	0.092 (0.076-0.11)	56.4
35	D-Tyr	D-Pya(4)	azaGly	Arg(Me)	3.6	0.12 (0.085-0.16)	0.12 (0.077-0.17)	ND[d]

[a] EC_{50} values of [Ca^{2+}] increasing activities of all peptide analogues were evaluated as ratios to that of metastin(45-54). [b] Concentration required to inhibit specific binding by 50%. [c] Residual ratio after incubation in mouse serum at 37 °C was evaluated. [d] Not determined.

4.3.2. Chemistry.

All peptides were synthesized by a standard Fmoc-based solid phase synthetic method (**Scheme 3**). Obtained crude peptides were purified to homogeneity with preparative HPLC. The purity of each peptide was ascertained by analytical HPLC, and the structure assignment was performed by MALDI-TOF MS.

4.3.3. Biological Activities.

Synthesized analogues were characterized in two assays for human KISS1R activity. The first was an intracellular calcium mobilization assay using FLIPR technology and the second was a binding assay. The calcium mobilization assay used CHO cells stably expressing recombinant human KISS1R and the binding assay used the membrane fraction of human KISS1R-expressing cells. The maximum response observed in the presence of a given compound was corrected for the baseline measurement and expressed as a percent of the corrected response to a maximal concentration of control metastin(45-54). Nonlinear regression of normalized data was performed to estimate the potencies (EC_{50}) of all compounds.

4.3.4. Serum Stability.

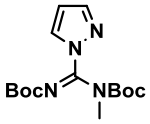
The stability against enzymatic hydrolysis of selected peptides was evaluated in mouse serum after incubation at 37 °C. The stability index of synthetic metastin derivatives was determined as the percent of compound remaining after 1 h incubation at an initial concentration of 0.1 mM.

Fmoc-Phe-Rink MBHA resin

↓ stepwise elongation (DCC/HOBt)

Fmoc-Orn(Mtt)-Phe-Rink MBHA resin

↓ 1) TFA/triisopropylsilane/DCM (1/5/94), r.t., 50 min

2)  (5 eq.), DIEA (10 eq.), DMF, r.t., 20 h

Fmoc-Arg(Boc_2,Me)-Phe-Rink-MBHA resin

↓ 1) 20% piperidine/DMF, r.t., 30 min

2) Fmoc-Leu, PyBOP, HOBt, DIEA, r.t., 2 h

Fmoc-Leu-Arg(Boc_2,Me)-Phe-Rink-MBHA resin

↓ 1) 20% piperidine / DMF, r.t., 30 min

2) CDI (4 eq.), THF, r.t., 2 h

3) hydrazine hydrate (8 eq.), r.t., 1 h

4) Fmoc-Phe (4 eq.), PyBrop (4 eq.), HOAt (4 eq.), DIEA (8 eq.), r.t., 2 h

5) stepwise elongation (DCC/HOBt)

6) TFA/thioanisole/*m*-cresol/trisopropylsilane/1,2-ethanedithiol (85/5/5/2.5/2.5), r.t., 2 h

7) HPLC purification

AA^{45}-AA^{46}-AA^{47}-Asn-Ser-Phe-azaGly-Leu-Arg(Me)-Phe-NH_2

Scheme 3. General synthetic route to compounds **36**-**58**

4.3.5. Decapeptide Analogues Substituted between Positions 45 and 47.

Decapeptide analogues with amino acid substitutions between positions 45 and 47 were designed and synthesized to obtain analogues showing improved biological activity and stability (**Figure 28**, **29**, **30**, **31**).

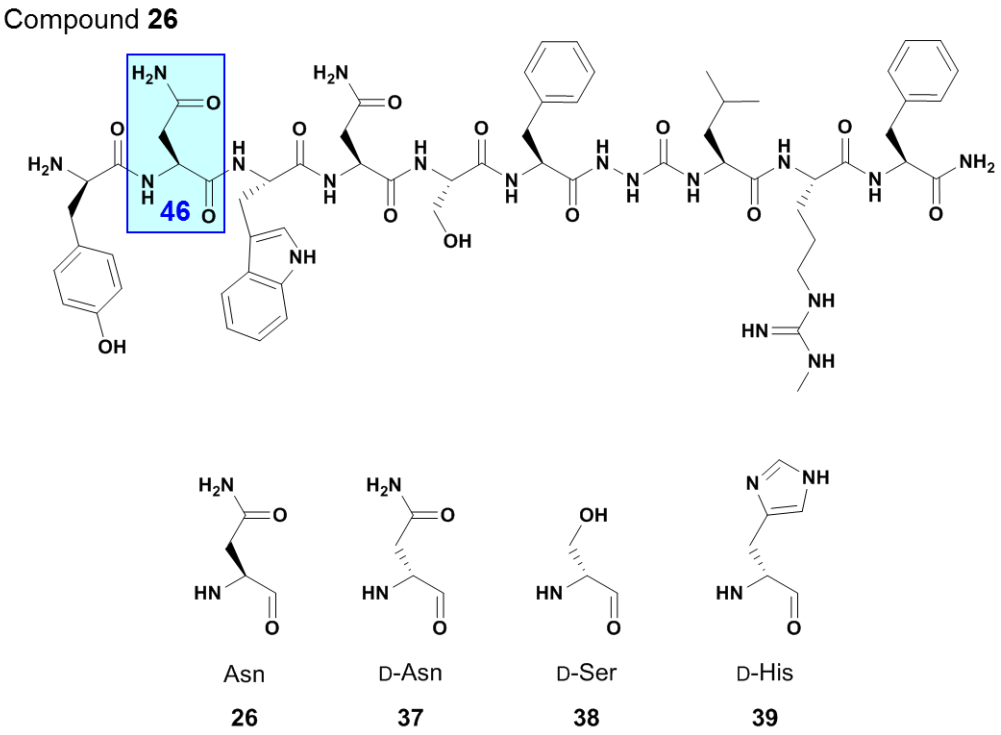


Figure 28. Structures of amino acids at position 46.

D-Amino acid substitutions were also expected to improve the pharmacokinetic profiles of metastin derivatives. In our previous studies, KISS1R recognition was shown to be unaffected by the structure of the *N*-terminal region of metastin(45-54), even if D-amino acid substitutions were made between positions 45 and 47. D-Asn^{46} derivative **37** exhibited nearly the same $[Ca^{2+}]_i$ mobilizing activity as that of the L-form analogue **26**, and showed more potent agonist activity than other derivatives with hydrophilic amino acids such as D-Ser **38** and D-His **39** (**Figure 28**, **Table 11**).

Analogues of **37** were synthesized that substituted Cha **41**, Thr **42**, Pya(4) **43**, and D-Trp **44** for Trp[47]; these were acceptable amino acids for Asn[46] derivatives (**Figure 29**, **Table 11**). A Phe[47] analogue **40**, which was expected to show improved serum stability due to a D-amino acid at the adjacent position, was also prepared.

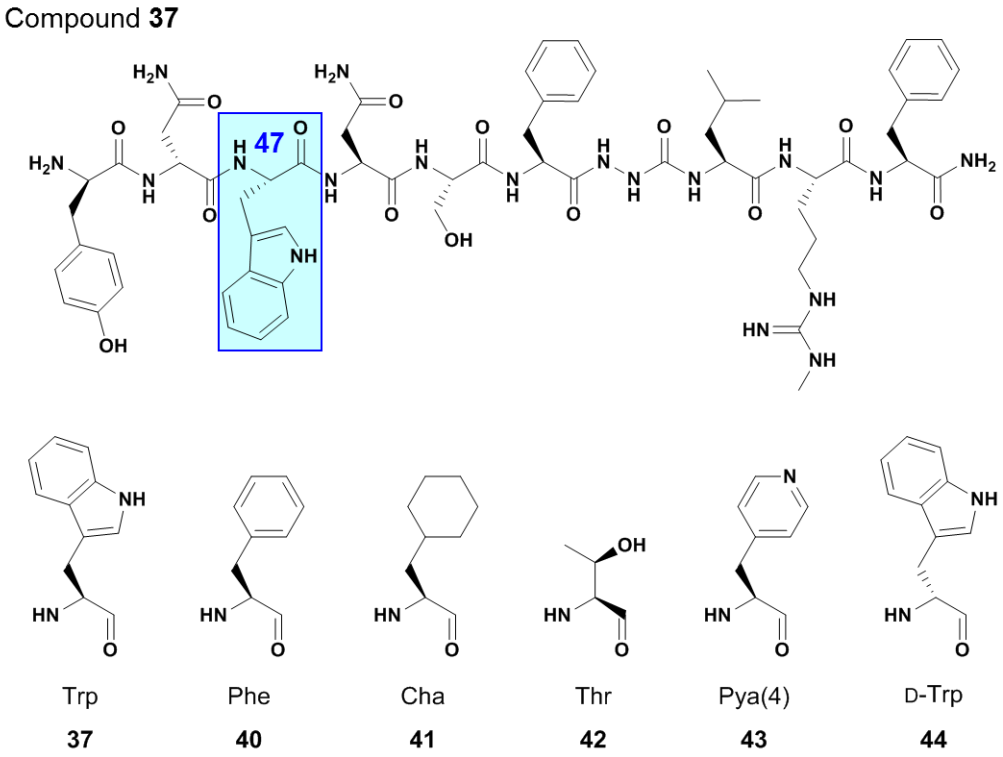


Figure 29. Structures of amino acids at position 47.

Analogue **40** possessed comparable *in vitro* agonist activity to metastin(45-54), however, the serum stability was decreased compared with that of Asn[46]-Trp[47] analogue **26**. Although Thr[47] analogue **42** showed excellent serum stability with a residual ratio of 65.8%, the agonist activity as well as the binding affinity was reduced. Cha and D-Trp analogues **41** and **44** also exhibited decreased $[Ca^{2+}]_i$ mobilizing activities. However, as Pya(4) analogue **43** showed excellent $[Ca^{2+}]_i$ mobilizing activity and receptor-binding affinities, compound **43** was selected as a candidate for the human KISS1R transfected

CHO (CHO/h175) cell growth inhibitory test described below.

Replacement of D-Tyr[45] with other amino acids including D-Ala **45**, D-Pya(3) **46**, **47**, and Tyr **48** resulted in moderate agonist activities that were not superior to the series of D-Tyr analogues (**Figure 30**, **31**, **Table 11**).

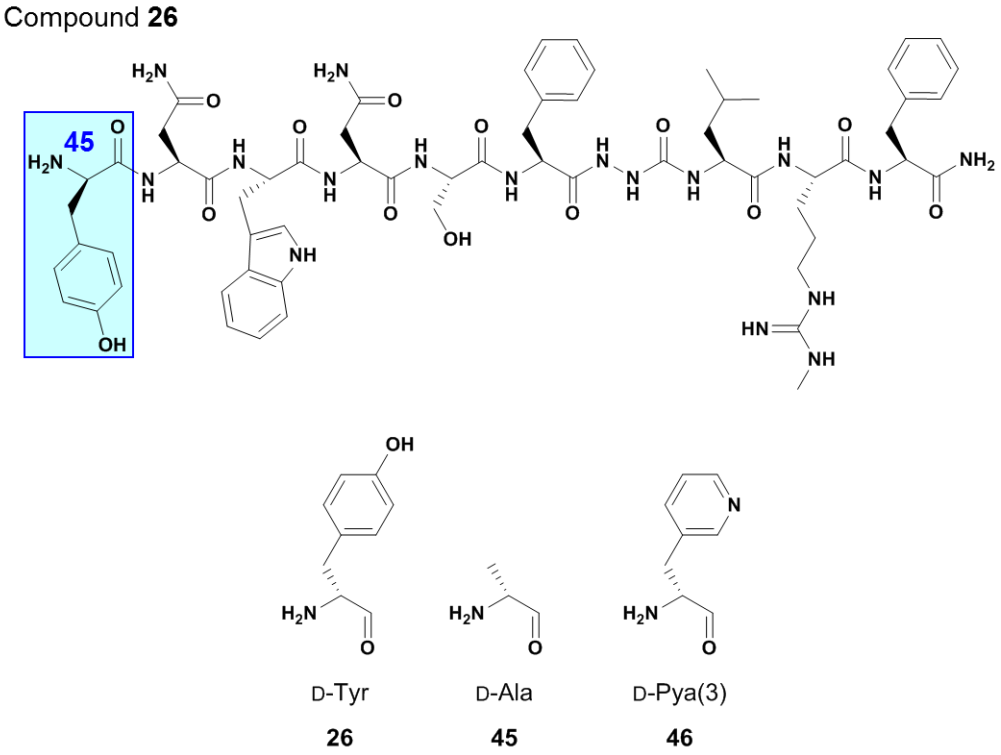


Figure 30. Structures of amino acids at position 45.

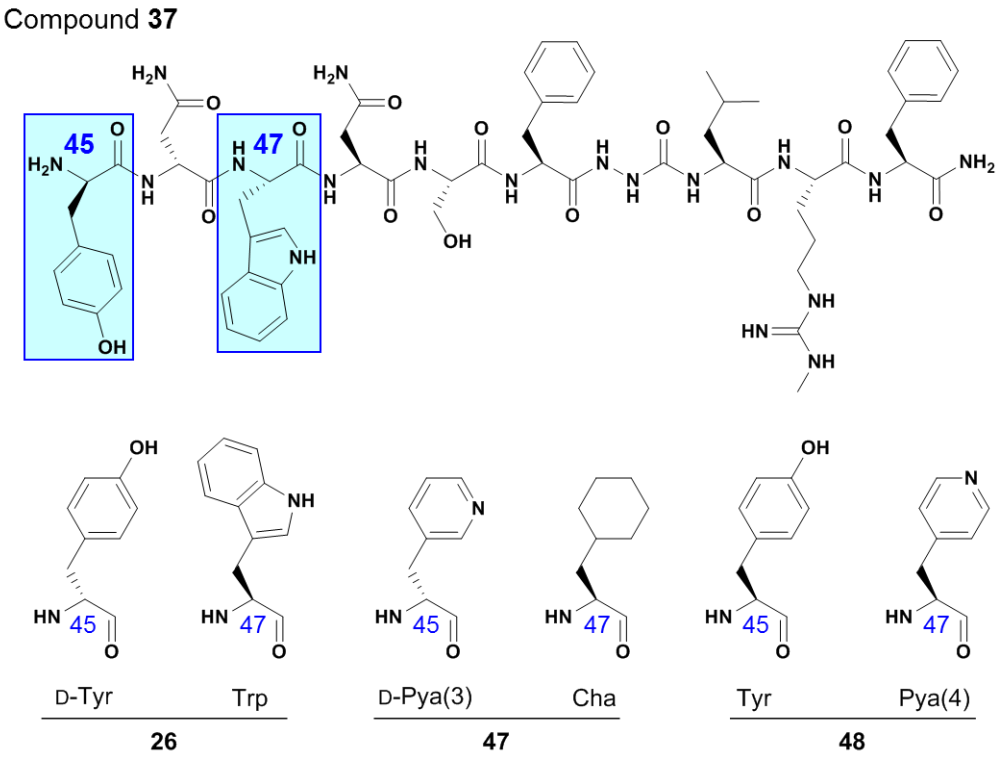


Figure 31. Structures of amino acids at positions 45 and 47.

Table 11. Biological activity of decapeptide metastin analogues.

H-AA^{45}-AA^{46}-AA^{47}-Asn-Ser-Phe-azaGly-Leu-Arg(Me)-Phe-NH_2

				agonist activity[a] EC_{50} ratio	affinities Ki nM (95% CI)[b]		stability in mouse serum
compound	AA^{45}	AA^{46}	AA^{47}	human	human	rat	residual ratio (%)[c]
26	D-Tyr	Asn	Trp	2.8	0.051 (0.040-0.064)	0.068 (0.048-0.096)	39.5
37	D-Tyr	D-Asn	Trp	2.6	0.062 (0.047-0.083)	0.042 (0.033-0.054)	ND[d]
38	D-Tyr	D-Ser	Trp	3.7	0.086 (0.068-0.11)	0.093 (0.050-0.17)	ND[d]
39	D-Tyr	D-His	Trp	6.2	0.033 (0.028-0.038)	0.043 (0.036-0.051)	ND[d]
40	D-Tyr	D-Asn	Phe	1.5	0.12 (0.074-0.19)	0.12 (0.061-0.22)	24.7
41	D-Tyr	D-Asn	Cha	17	0.055 (0.037-0.082)	0.076 (0.036-0.16)	ND[d]
42	D-Tyr	D-Asn	Thr	7.3	0.29 (0.18-0.47)	0.25 (0.14-0.44)	65.8
43	D-Tyr	D-Asn	Pya(4)	1.7	0.11 (0.089-0.14)	0.095 (0.067-0.13)	ND[d]
44	D-Tyr	D-Asn	D-Trp	8.6	0.052 (0.042-0.065)	0.070 (0.054-0.090)	ND[d]
45	D-Ala	Asn	Trp	4.9	0.51 (0.34-0.77)	0.52 (0.35-0.76)	ND[d]
46	D-Pya(3)	Asn	Trp	2.3	0.12 (0.11-0.14)	0.14 (0.10-0.18)	ND[d]
47	D-Pya(3)	D-Asn	Cha	10	0.20 (0.16-0.25)	0.18 (0.13-0.27)	ND[d]
48	Tyr	D-Asn	Pya(4)	2.7	0.21 (0.16-0.27)	0.16 (0.14-0.19)	ND[d]

[a] EC_{50} values of [Ca^{2+}] increasing activities of all peptide analogues were evaluated as ratios to that of metastin(45-54). [b] Concentration required to inhibit specific binding by 50%. [c] Residual ratio after incubation in mouse serum at 37 °C was evaluated. [d] Not determined.

4.4. Design and Synthesis of Nonapeptide KISS1R Agonists Substituted at Positions 46 and 47.

4.4.1. Deamidation of Asn-containing Peptide.

Asn-containing peptides have been shown to undergo spontaneous rearrangement (i.e., Asn conversion to Asp and β-Asp) in aqueous environments (**Figure 32**).[64-65] Therefore, deletion of Asn at position 46 was intended to avoid inactivation of the peptide through chemical isomerization.

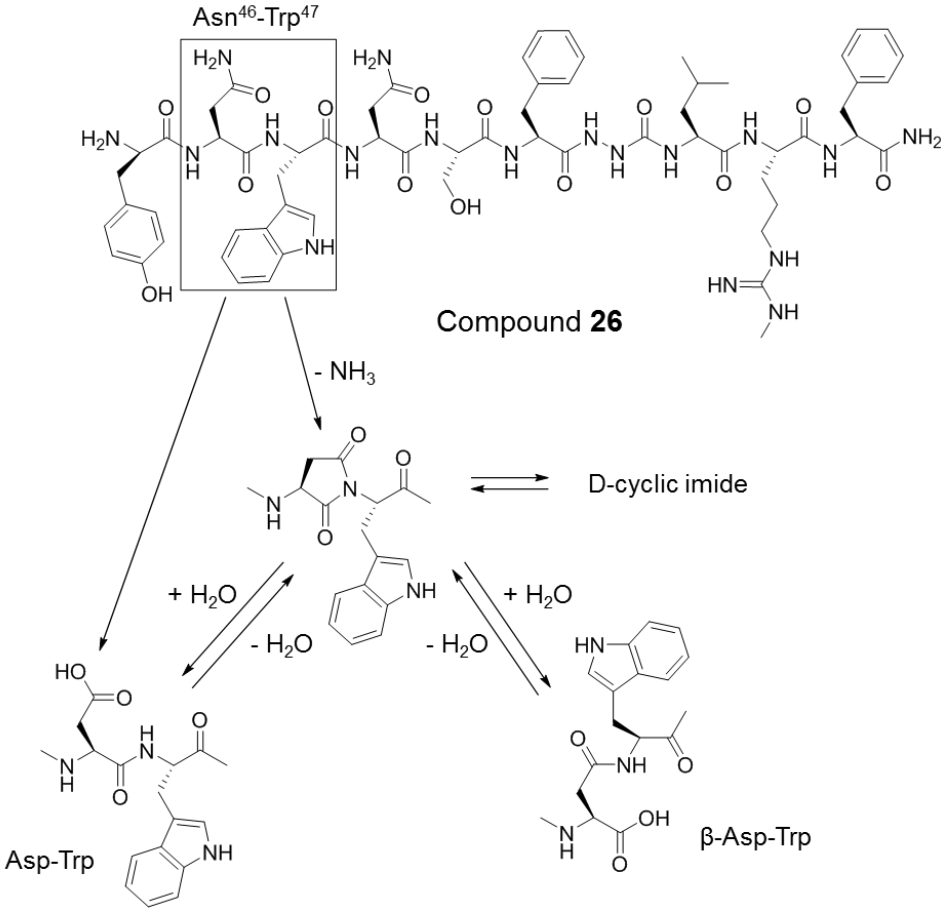


Figure 32. Proposed scheme of deamidation reaction of an Asn-containing metastin analogue **26** in aqueous solution.

Table 12. Biological activity of decapeptide and nonapeptide metastin analogues.

H-AA^{45}-AA^{46}-AA^{47}-Asn-Ser-Phe-azaGly-Leu-Arg(Me)-Phe-NH_2

compound	AA^{45}	AA^{46}	AA^{47}	agonist activity[a] EC_{50} ratio	affinities Ki nM (95% CI)[b]		stability in mouse serum
				human	human	rat	residual ratio (%)[c]
metastin(45-54)				1.0	0.039 (0.032-0.047)	0.057 (0.035-0.093)	ND[d]
26	D-Tyr	Asn	Trp	2.8	0.051 (0.040-0.064)	0.068 (0.048-0.096)	39.5
49		D-Tyr	Trp	2.4	0.044 (0.037-0.052)	0.059 (0.045-0.076)	43.4

[a] EC_{50} values of [Ca^{2+}] increasing activities of all peptide analogues were evaluated as ratios to that of metastin(45-54). [b] Concentration required to inhibit specific binding by 50%. [c] Residual ratio after incubation in mouse serum at 37 °C was evaluated. [d] Not determined.

The biological activities of the synthesized nonapeptides are summarized in **Table 12** and **13**. The Asn[46]-deleted analogue **49** exhibited a slight increase in binding affinity compared with decapeptide analogue **26**. The phenol group of Tyr[45] was thought to be important for receptor recognition, but not to play a pivotal role (**Table 12**).

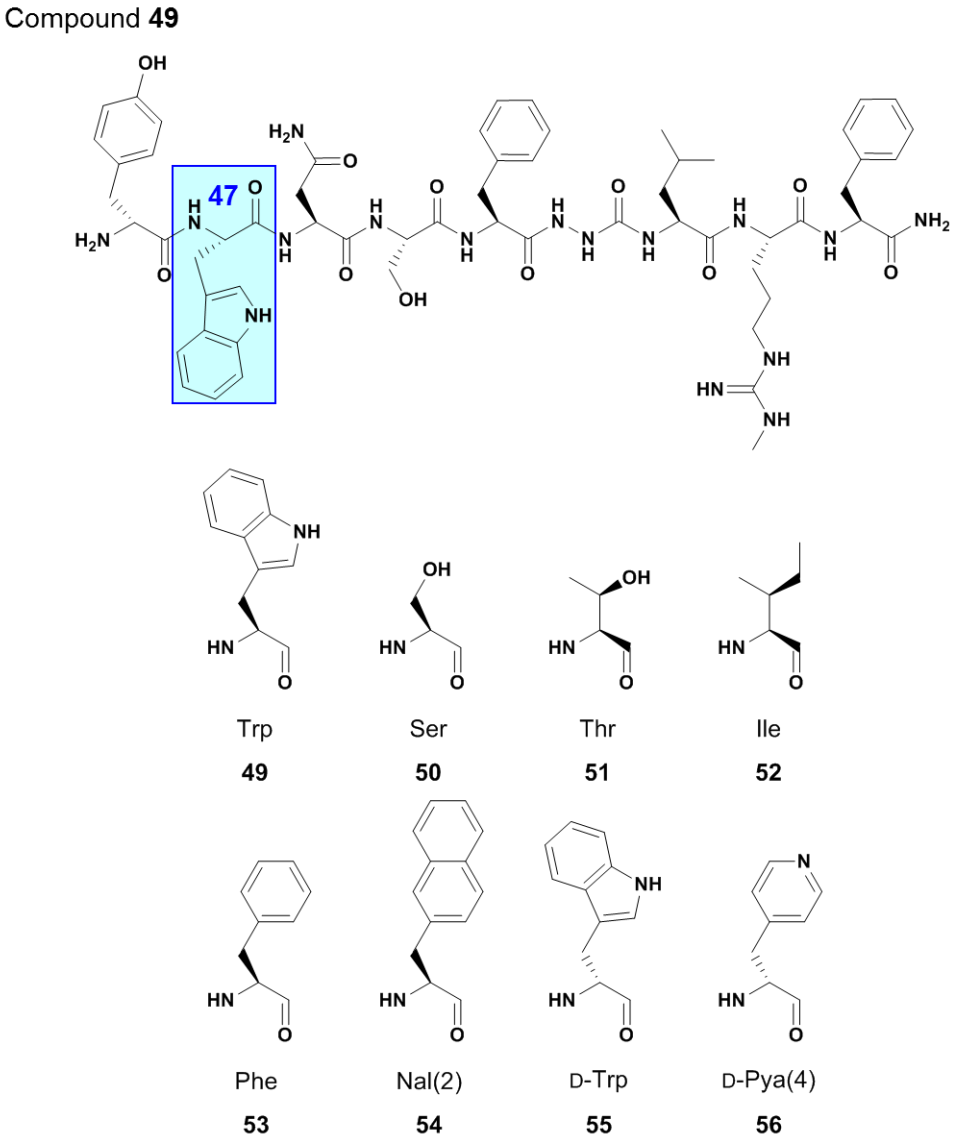


Figure 33. Structures of amino acids at position 47.

4.4.2. Nonapeptide Analogues Substituted at Positions 46 and 47.

D-Amino acid substitutions between positions 45 and 47 in metastin(45-54) were

acceptable for maintenance of the *in vitro* activities of decapeptides **26**, **34**, **37,** and **48**. Positions 45-47 were considered to be flexible for receptor recognition. This was supported by data showing that modifications at the Tyr^{45}-Trp^{47} positions **51-58** of the nonapeptide analogues resulted in small decreases in biological activity (**Figure 33**, **34**).

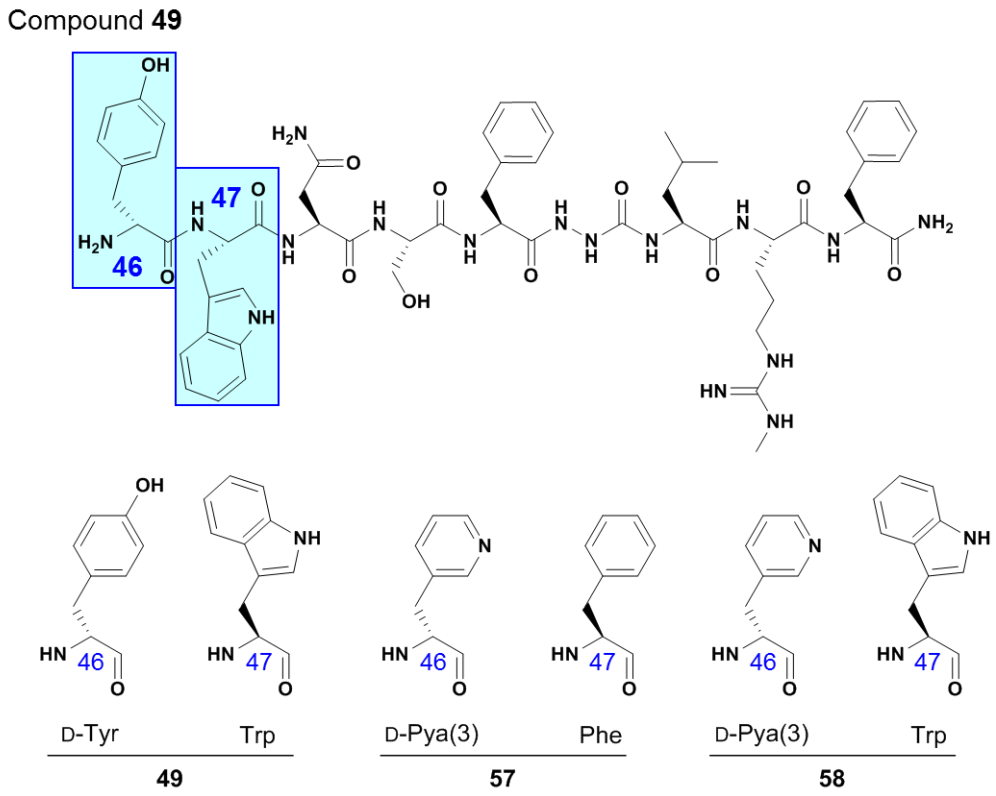


Figure 34. Structures of amino acids at positions 46 and 47.

Nonapeptide analogues which substituted for Trp^{47} with Thr **51**, Ile **52**, Phe **53**, and Nal(2) **54** had three- to six-fold lower agonist activity than Trp analogue **19** (**Table 13**). On the other hand, D-amino acid substitutions at the Trp^{47} position, such as D-Trp **55** and D-Pya(4) analogue **56**, did not hamper the potent agonist activities. D-Pya(4) analogue **56** displayed good agonist activity *in vitro* and high binding affinity to the receptor, which was similar to that of metastin(45-54).

Table 13. Biological activity of nonapeptide metastin analogues.

H-AA[46]-AA[47]-Asn-Ser-Phe-azaGly-Leu-Arg(Me)-Phe-NH_2

compound	AA^{46}	AA^{47}	agonist activity[a] EC_{50} ratio human	affinities Ki nM (95% CI)[b] human	affinities Ki nM (95% CI)[b] rat	stability in mouse serum residual ratio (%)[c]
49	D-Tyr	Trp	2.4	0.044 (0.037-0.052)	0.059 (0.045-0.076)	43.4
50	D-Tyr	Ser	61	0.21 (0.15-0.31)	0.30 (0.22-0.40)	ND[d]
51	D-Tyr	Thr	8.9	0.20 (0.16-0.25)	0.24 (0.18-0.32)	ND[d]
52	D-Tyr	Ile	14	0.12 (0.090-0.17)	0.13 (0.089-0.19)	ND[d]
53	D-Tyr	Phe	7.7	0.086 (0.062-0.12)	0.15 (0.11-0.23)	ND[d]
54	D-Tyr	Nal(2)	6.4	0.032 (0.027-0.038)	0.051 (0.043-0.061)	ND[d]
55	D-Tyr	D-Trp	4.5	0.065 (0.057-0.075)	0.11 (0.072-0.16)	ND[d]
56	D-Tyr	D-Pya(4)	4.8	0.089 (0.072-0.11)	0.10 (0.074-0.14)	54.9
57	D-Pya(3)	Phe	5.8	0.20 (0.16-0.24)	0.28 (0.21-0.38)	ND[d]
58	D-Pya(3)	Trp	8.7	0.11 (0.067-0.18)	0.13 (0.075-0.23)	ND[d]

[a] EC_{50} values of [Ca^{2+}] increasing activities of all peptide analogues were evaluated as ratios to that of metastin(45-54). [b] Concentration required to inhibit specific binding by 50%. [c] Residual ratio after incubation in mouse serum at 37 °C was evaluated. [d] Not determined.

Furthermore, this D-amino acid substitution improved the serum stability of compound **56**, as indicated by the residual ratio of 54.9% in mouse serum at 37 °C. Compound **56** with good water solubility of 1 mM met the criteria initially set as tool peptide which was expected to work *in vivo*.

Replacement of D-Tyr^{46} with D-Pya(3) in analogues **57** and **58** introduced a hydrophilic/aromatic side chain for improvement of stability and solubility and retained moderate agonist activities, although no improvement over the series of D-Tyr analogues was observed. Consequently, substitution of *N*-terminal Tyr/D-Tyr residues in decapeptide and nonapeptide metastin analogues was determined to be the best choice, although further investigations employing other aromatic amino acid substitutions may provide even better results.

4.5. Effect of Metastin Analogues on the Growth of KISS1R Transfected CHO Cells.

We previously reported that metastin inhibited human KISS1R transfected CHO (CHO/h175) cell growth and colony formation.[66] To examine the ability of metastin analogues to inhibit KISS1R-mediated cell growth, we compared the inhibitory activity of compounds **22**, **26**, **32**, **43**, and **56** on CHO/h175 cell proliferation. Metastin significantly inhibited growth of CHO/h175 cells in liquid culture (IC_{50} = 21.2 nM) and inhibited colony formation in semi-solid agarose at a concentration of 5 μM.

Table 14. Inhibition of CHO/h175 cell growth by metastin and metastin analogues.

compound	IC_{50} (nM)[a]
metastin	21.2
metastin(45-54)	8510
22	87.0
26	9.67
32	18.3
43	41.2
56	8.94

[a] IC_{50} values of cell growth inhibitory activity in hOT7T175-expressed CHO cells cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% dialyzed fetal bovine serum (FBS).

The *N*-terminally truncated analogue metastin(45-54) showed inhibitory activity against CHO/h175 cell growth, however, the IC_{50} value was decreased (IC_{50} = 8510 nM) compared with native metastin (**Table 14**). But the metastin analogues had greatly

improved antiproliferative activity compared with metastin(45-54). The lead compound with D-Tyr^{45} and Arg(Me)^{53} **22** slowed cell growth in CHO/h175 cells with an IC_{50} of 87.0 nM, which suggested that the functional activity and the biological stability of compound **22** were improved by several key amino acid substitutions. Substitutions at positions 47 and 51 revealed that azaGly analogues with Trp^{47} **26** and Pya(4)^{47} **32** showed further improvements in growth inhibitory activities together with enhancement of serum stability. The IC_{50} values of peptides **26** and **32** were 9.67 and 18.3 nM, respectively, which is up to 40-times more potent than metastin(45-54).

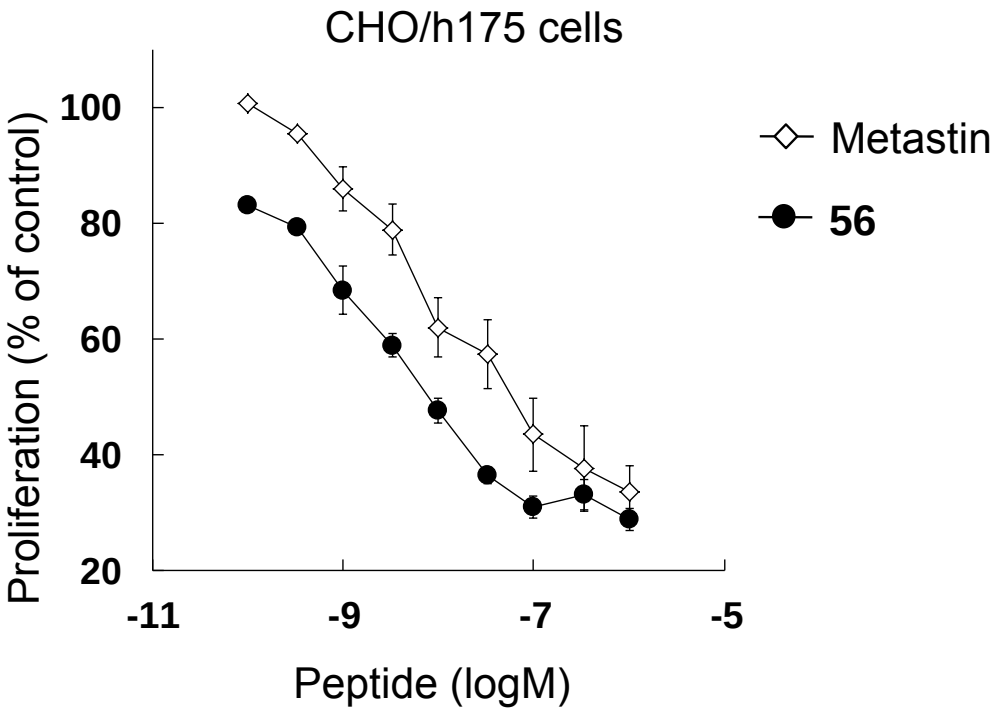


Figure 35. Inhibition of CHO/h175 cell growth by metastin and compound **56**.

Among the series of decapeptide metastin analogues, D-Asn^{46}-Pya(4)^{47} derivative **43** was tested because it showed the lowest EC_{50} ratio. However, the IC_{50} value of growth inhibitory activity was not improved (IC_{50} = 41.2 nM) compared with **26** and **32**. This

might be due to the lower metabolic stability of compound **43** compared with those of the Asn^{46} derivatives **26** and **32**.

Similar to the decapeptide analogues, the representative nonapeptide analogue **56** inhibited cell growth in CHO/h175 cells with an IC_{50} of 8.94 nM (**Table 14**, **Figure 35**). We believe that several key amino acid substitutions in compound **56** play an important role in the improved functional activity and enzymatic stability.

4.6. Ovulation Induction Activity in Immature Female Wistar Rats.

The first *in vivo* pharmacodynamic experiment was carried out to obtain proof-of-concept using an ovulation induction model in immature female Wistar rats[9] that were primed with equine chorionic gonadotropin (eCG, 10 IU/rat). A single subcutaneous dose of human CG (hCG; 10 IU/rat), which was used as a positive control, was sufficient to trigger ovulation in all of the animals (n = 5), with a mean of 26.0 oöcytes per animal. The number of oöcytes obtained is shown in **Figure 36** and the minimum effective dose of metastin was 100 nmol/kg.

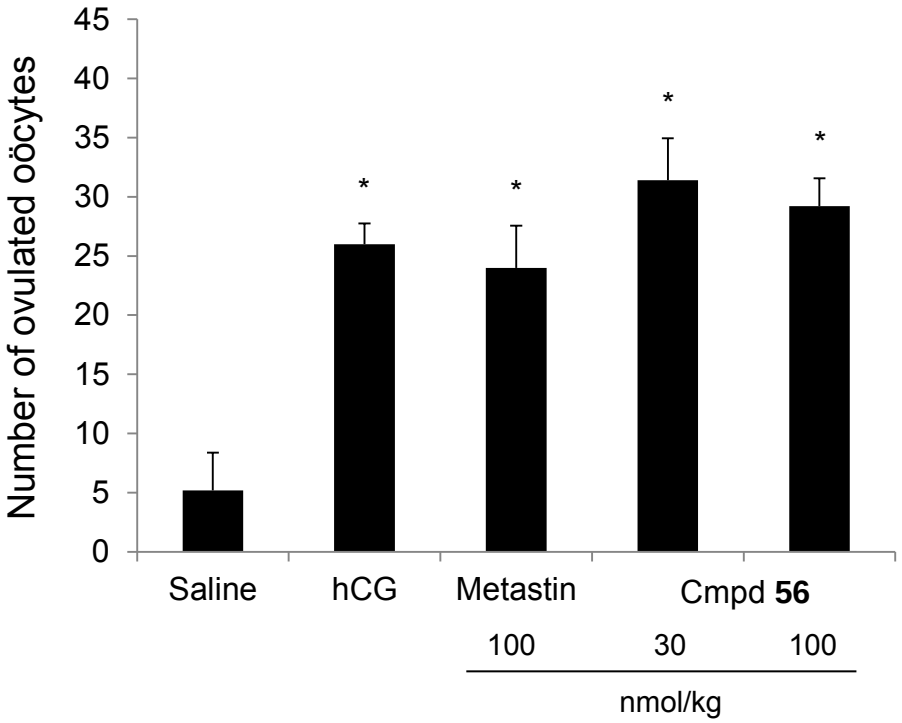


Figure 36. Induction of ovulation in immature rats by metastin and compound **56** compared to saline and hCG (10 IU/rat)[5].

When compound **56** was administered at 30 or 100 nmol/kg, the average number of oöcytes was 31.4 or 29.2, respectively, which indicated that the efficacy of compound **56** was essentially equivalent to that of hCG. In summary, a short-length peptide **56** showed ovulation induction activity *in vivo* comparable to full-length metastin, confirming proof-of-concept.

By comparison, metastin(45-54), a short-length peptide that has strong agonist activity, did not show ovulation induction activity compared with compound **56** (**Figure 37**). This suggests that *in vivo* efficacy of compound **56** is based not only on good agonist activity, but also on biologic stability.

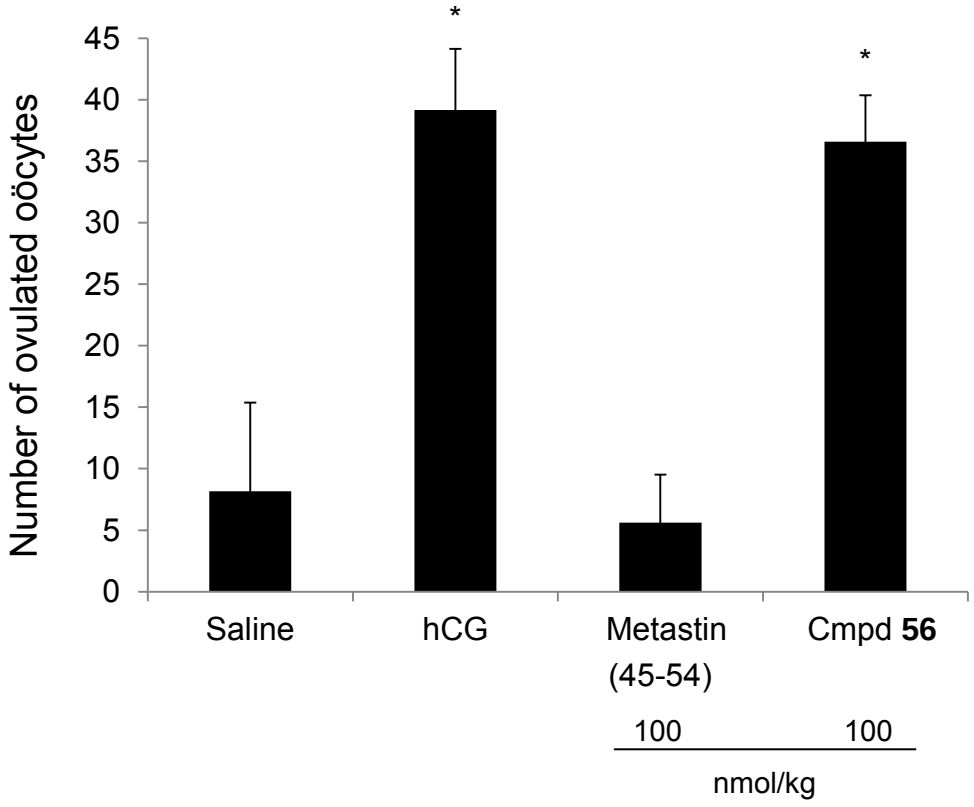


Figure 37. Induction of ovulation in immature rats by metastin(45-54) and compound **56** at a dose of 100 nmol/kg compared to saline and hCG (10 IU/rat)[5].

4.7. Efficacy of KISS1R Agonists for Decreasing Testosterone Levels in Intact Male Rats.

As previously reported,[67,68] the continuous administration of metastin or metastin(45-54) to normal male rats temporarily increased their testosterone levels followed by a subsequent decrease to below normal levels. Our lead compound **22** and the optimized peptide **56** with nine amino acid residues including azaGly[51] were subjected to an intact male rat assay. In this assay, plasma testosterone levels were determined at steady state after 6 days of continuous administration of each metastin analogue. These results provided a direct measure of the *in vivo* efficacy of compounds **22** and **56** in the suppression of testosterone levels in an intact animal model using male Crl:CD(SD) rats (**Figure 38**).

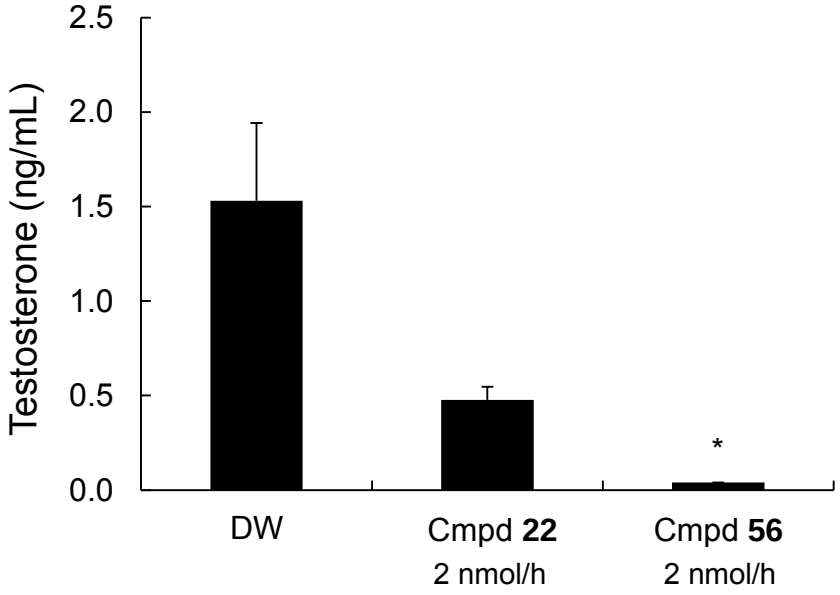


Figure 38. Plasma testosterone suppression in intact male rats by decapeptide compound **22** and optimal nonapeptide **56** at a dose of 2 nmol/hr compared to a distilled water (DW) control.

The limit of plasma testosterone measurement in this RIA was 0.04 ng/mL; values below 0.04 ng/mL were estimated as 0.04. As shown in **Figure 38**, compound **56** suppressed testosterone levels lower than the limit of detection (0.04 ng/mL), demonstrating that compound **56** was more effective at lowering testosterone levels than compound **22**.

In a second experiment using male Copenhagen rats (**Figure 39**), both 2 nmol/h administration of compound **56** and 6 nmol/h administration of metastin attenuated plasma testosterone levels.

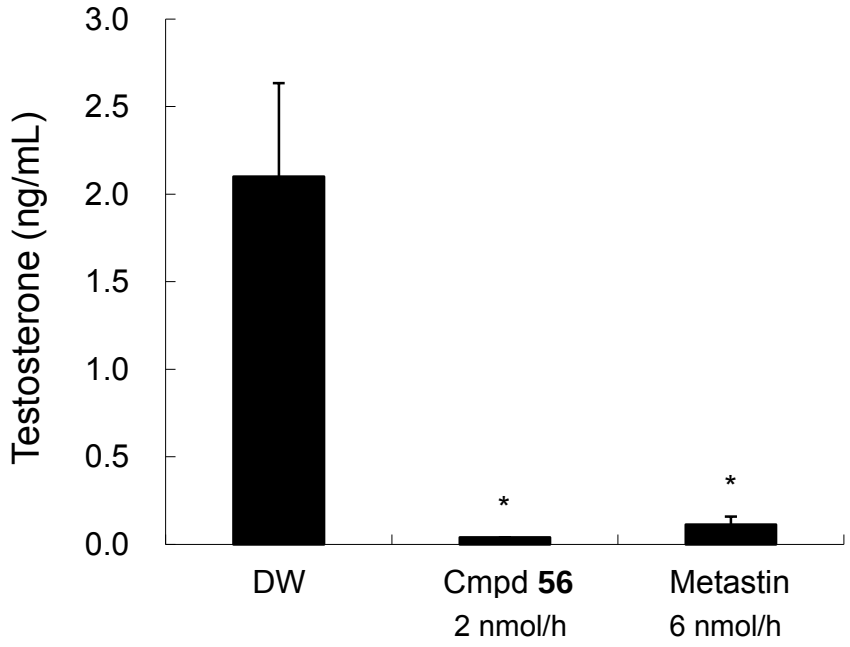


Figure 39. Plasma testosterone suppression by continuous administration of compound **56** or metastin in male rats at doses of 2 or 6 nmol/h, respectively compared to a distilled water (DW) control.

The plasma concentrations of the short-length peptide **56** (2 nmol/h) and metastin (6 nmol/h) in this study were 0.95 and 4.07 nM, respectively. The *in vivo* efficacy of compound **56** might be based on its enzymatic stability in plasma. In addition, the moderate bioavailability (40.4 ± 4.9%) of compound **56** after subcutaneous administration at 1 mg/kg in IGS/SD rats might be explained by resistance to enzymatic degradation in subcutaneous tissue.

4.8. Continuous Administration of Compound 56 and Leuprolide Acetate in Male Rats for 13 Days.

Compound **56** and leuprolide acetate[69] were administered to male rats to compare plasma testosterone reducing efficacy. Plasma testosterone levels were determined after 6 and 13 days of continuous administration.

Continuous administration of compound **56** and leuprolide acetate at a dose of 1 nmol/h for 13 days significantly suppressed testosterone in all rats (**Figure 40**). The plasma testosterone levels in rats receiving compound **56** decreased faster than in those receiving leuprolide acetate. Six-day dosing of compound **56** dramatically reduced plasma testosterone levels in rats; two of five rats showed plasma testosterone concentrations below the limit of detection (0.04 ng/mL). These findings suggest that the mechanisms of action for reducing plasma testosterone are different between metastin and GnRH agonists.

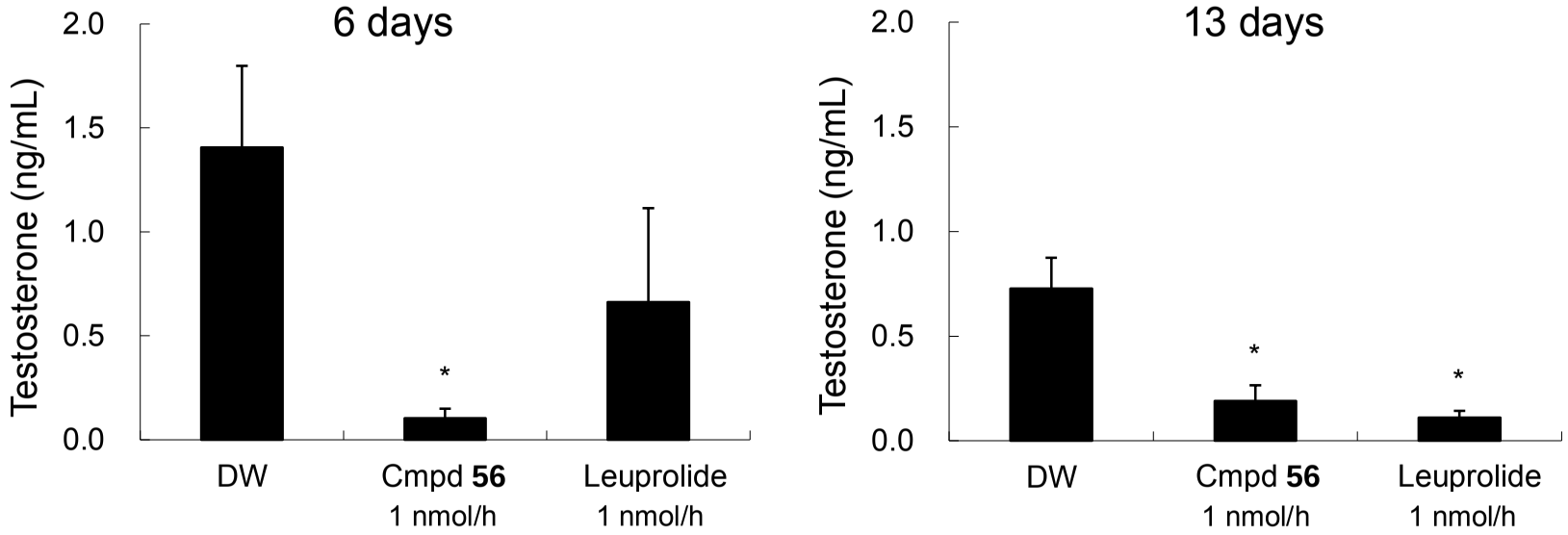


Figure 40. Plasma testosterone suppressive activities by continuous administration of compound **56** and leuprolide acetate in male rats at a dose of 1 nmol/h compared to a distilled water (DW) control.

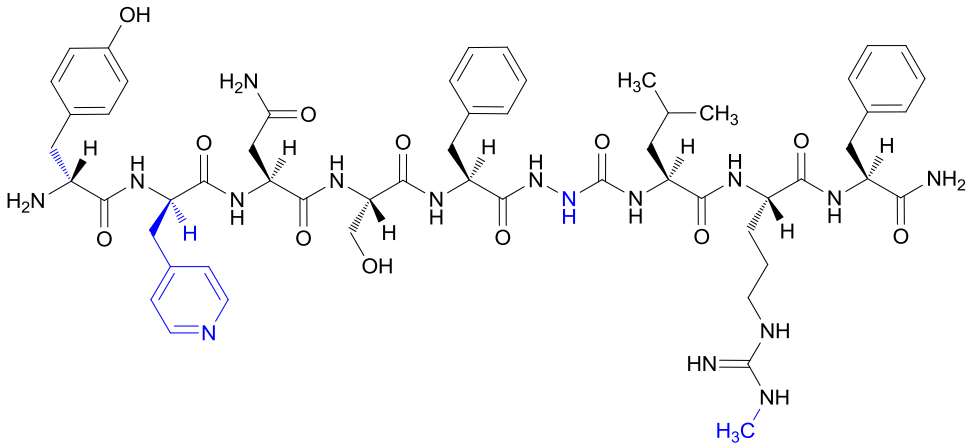
4.9. Conclusion of Chapter 4.

In order to identify metastin analogues with improved metabolic stability and enhanced potency *in vivo*, a series of metastin(45-54) analogues containing three key replacements – *N*-terminal D-Tyr residue, azaGly^{51}, and Arg(Me)^{53} – were synthesized and studied *in vitro* and *in vivo*. Deletion of Asn^{46} and the substitution of Trp^{47} with different amino acids, together with the substitutions mentioned above, produced highly potent and long-acting analogues suitable for *in vivo* study (**Figure 41**).

The results presented here demonstrate that both agonist activity and metabolic stability were important for performance of these peptides *in vivo*. The testosterone-suppressive activities of compound **56** may indicate a greater contribution of its improved metabolic stability, which resulted in enhanced performance of this metastin analogue *in vivo*.

Continuous dosing of compound **56** at 1 nmol/h in male rats demonstrated testosterone suppressive efficacy as potent as that of leuprolide acetate. Leuprolide acetate has been known to down-regulate and desensitize the GnRH receptor, followed by inhibition of testosterone secretion. However, recent studies have suggested that KISS1R agonists may operate by a different mechanism to attenuate testosterone levels in the HPG axis.[70] Compound **56** is the first short-length KISS1R agonist to possess testosterone suppressive activity comparable to that of leuprolide acetate *in vivo*.

Compound **56** D-Tyr-D-Pya(4)-Asn-Ser-Phe-azaGly-Leu-Arg(Me)-Phe-NH_2



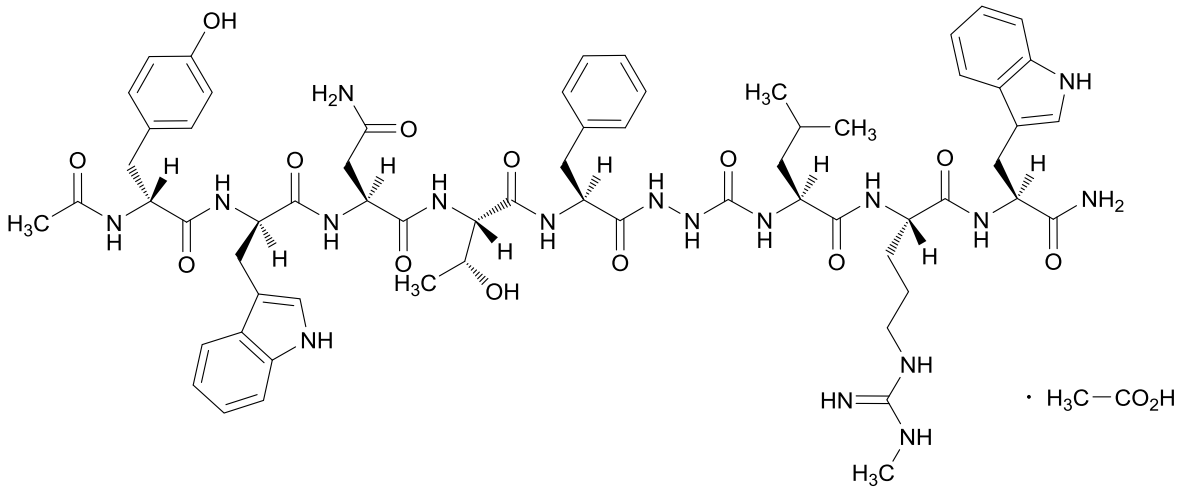
	agonist activity[a]		binding affinity[b]				
	human		human		rat		stability in mouse serum
compound	EC_{50} (nM)	**EC_{50} ratio**	Ki (nM)	**Ki ratio**	Ki (nM)	**Ki ratio**	**Residual ratio (%)[c]**
metastin(45-54)	0.049	**1.0**	0.039	**1.0**	0.057	**1.0**	**ND[d]**
56	0.27	**5.5**	0.089	**2.3**	0.1	**1.8**	**54.9**

[a] Agonist activities of all peptide analogues were evaluated in a functional assay of KISS1R (hOT7T175), an intracellular calcium mobilization assay using fluorometric imaging plate reader technology. EC_{50} values of all peptide analogues were calculated using sigmoidal dose response curves. EC_{50} ratio means ratio of each peptide EC_{50} value to that of metastin(45-54). [b] Concentration required to inhibit specific binding by 50%. Ki ratio means ratio of each peptide Ki value to that of metastin(45-54). [c] Residual ratio after incubation in mouse serum for 1 h at 37 °C. [d] Not determined.

Figure 41. Biological profiles of metastin(45-54) and compound **56**.

Compound **56** has led to the elucidation of investigational analogues TAK-683 and TAK-448 (**Figure 42**), both of which have undergone clinical evaluation. Our results suggest that short-length KISS1R agonists can be efficacious in humans, leading to development of novel and effective investigational candidates for the therapy of sex-hormone-dependent diseases such as prostate cancer.

TAK-683



TAK-448

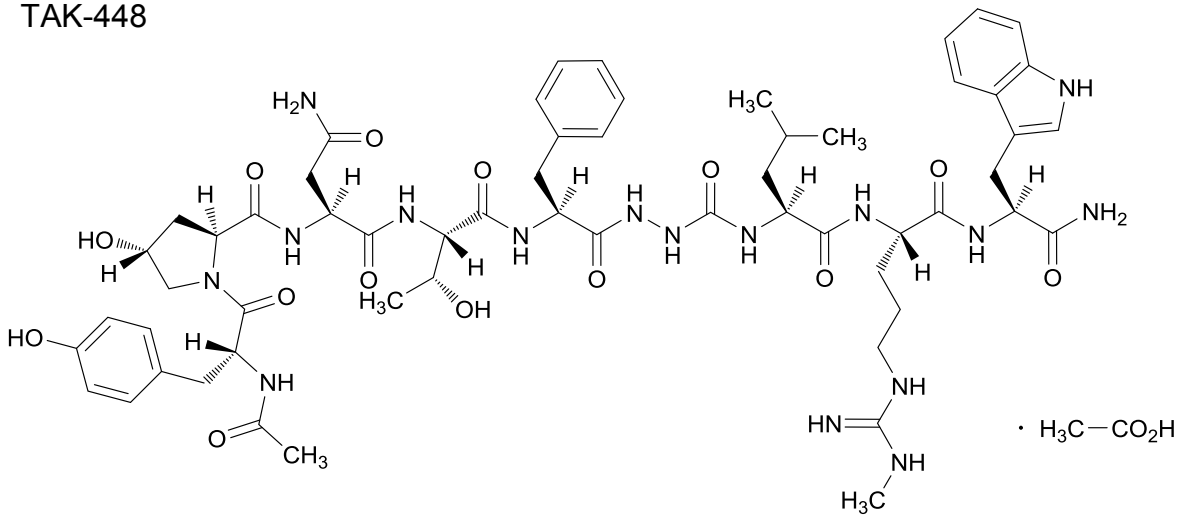


Figure 42. Chemical structures of investigational compounds TAK-683 and TAK-448.

Chapter 5 Concluding Remarks

In this study compound **56** with highly potent agonist activity and biological stability was successfully designed and synthesized from a lead peptide metastin(45-54) (**Figure 43**).

N-terminal truncated human metastin(45-54), Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2, has 3 to 10 times more potent KISS1R agonist activity than metastin. Although short-length analogues of metastin have the potential to possess therapeutic properties, their susceptibility to enzyme cleavage limits their utility. Actually metastin(45-54) was rapidly metabolized in mouse serum, and the half-life was less than 1 min mainly due to sensitive Tyr and Arg residues.

The metabolites formed during the incubation in serum were identified by HPLC-ESI-MS coupling experiments; major cleavage sites were Tyr^{45}-Asn^{46} and Arg^{53}-Phe^{54}. Arg-containing peptides are known to be good substrates for trypsin-like proteases; therefore, we designed and synthesized a series of short-length KISS1R agonists to examine their agonist activity and trypsin resistance. Among analogues with substitutions of arginine at position 53, N^{ω}-methylarginine analogue **8** showed 3-fold more potent agonist activity compared with metastin(45-54). Furthermore, analogue **8** was shown to resist trypsin cleavage between positions 53 and 54. This substitution may be useful in the development of other Arg-containing peptides for which the avoidance of cleavage is desired (**Chapter 2**).

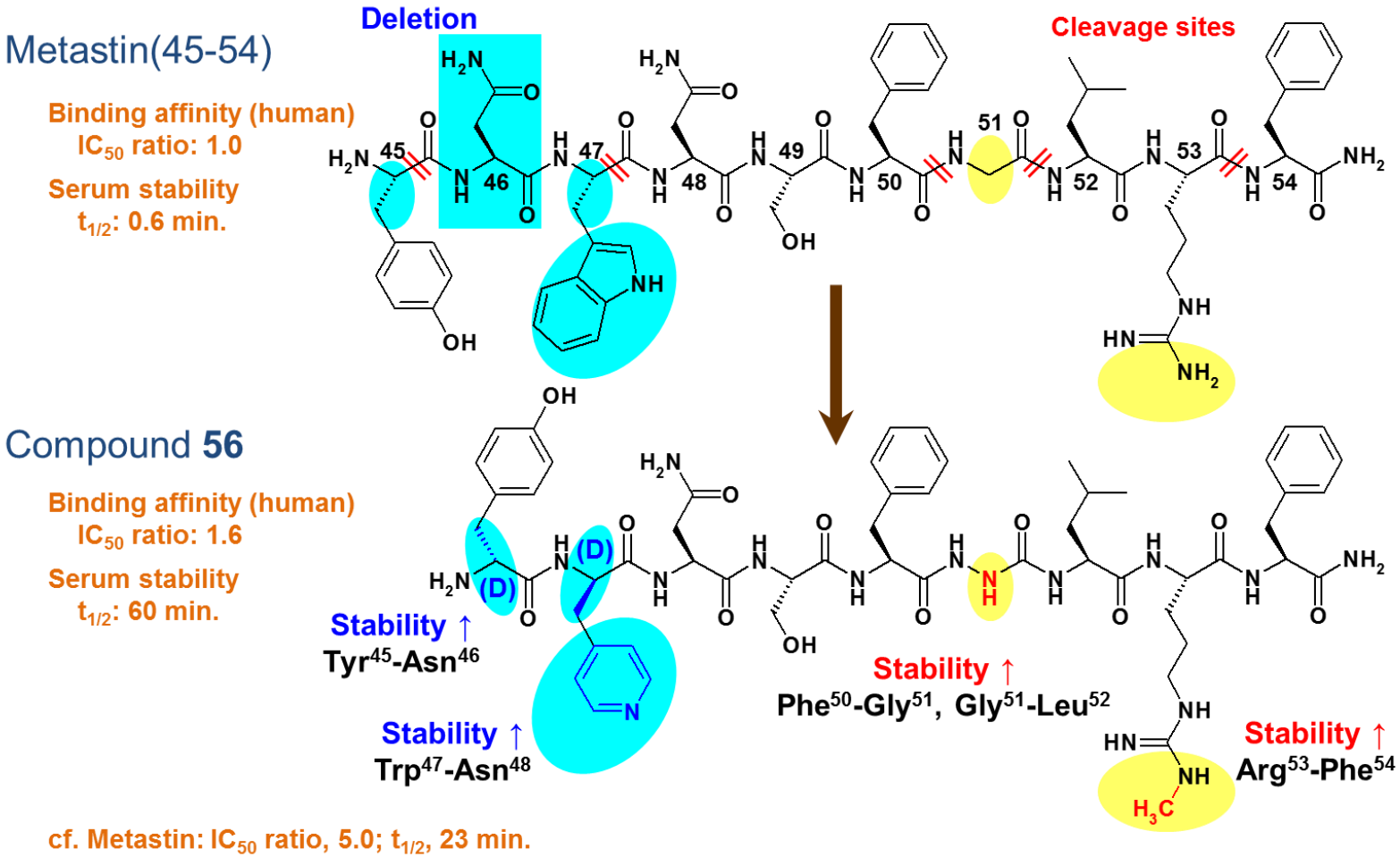


Figure 43. Summary of optimization for biologically potent and stable KISS1R agonist.

There remained a great demand for more stable analogues with other amino acid replacements at fragile sites to improve their pharmacological properties by maintaining or improving agonist activity and serum stability. Incorporation of D-Tyr at position 45 showed acceptable activity and avoided *N*-terminal degradation. Mouse plasma stability experiments showed that [D-Tyr^{45},Arg(Me)^{53}]metastin(45-54) **22** was apparently resistant to proteolysis after a 1 h incubation in mouse serum, even though several metabolites were observed in the ESI-MS spectra including metastin(45-47), (45-50), (45-51), and (48-54), possibly due to cleavage by chymotrypsin-like proteases, NEP, and MMP-9. We hypothesized that more potent and long-acting analogues could be developed if other degradative pathways were successfully inhibited by specific replacement at positions 47, 48, 50, and 51.

To examine this hypothesis, we first synthesized a series of analogues with substitution of residues at positions 50 and 51. Preliminary data (not shown) suggested that amino acid substitutions at Phe^{50} and Gly^{51} preserved receptor recognition. Therefore, we designed and synthesized amide bond isosteres and aza amino acid additions between positions 50 and 51 without altering their side chain moieties. This was expected to improve agonist activity and avoid enzymatic degradation by changing the overall conformation of compound **22**. Replacement of Gly with azaGly in which the α-carbon is replaced with a nitrogen atom at position 51 improved the stability of amide bonds between Phe^{50}-Gly^{51} and Gly^{51}-Leu^{52} as determined by *in vitro* mouse serum stability studies.

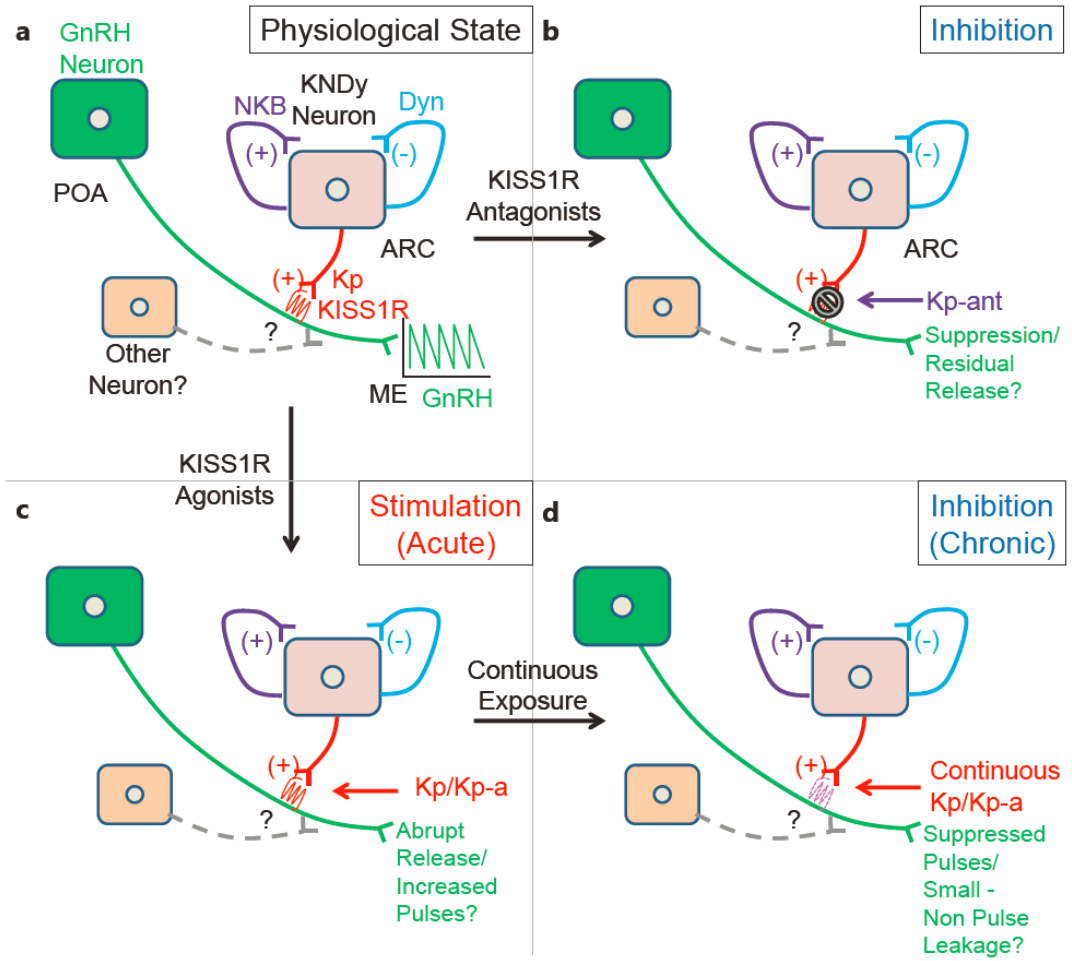
Substitution for tryptophan at position 47 with other amino acids produced analogues that were highly stable in mouse serum. D-Trp^{47} analogue **34** showed not only high

metabolic stability but also excellent KISS1R agonist activity (**Chapter 3**).

Further amino acid replacements at positions 45-47 led to enhancement of KISS1R agonist activity and metabolic stability. *N*-Terminal truncation resulted in a stable nonapeptide, [D-Tyr^{46},D-Pya(4)^{47},azaGly^{51},Arg(Me)^{53}]metastin(46-54), compound **56**, which displayed KISS1R binding affinities comparable to metastin(45-54) and had improved serum stability.

Continuous dosing of compound **56** demonstrated testosterone suppressive efficacy as potent as that of leuprolide acetate in male rats. Leuprolide acetate has been known to down-regulate and desensitize the GnRH receptor, followed by inhibition of testosterone secretion. However, recent studies have suggested that KISS1R agonists may operate by a different mechanism to attenuate testosterone levels in the HPG axis (**Figure 44**).

In physiologically stable conditions, GnRH pulses are generated from GnRH neural terminals, to which kisspeptin/neurokinin B/dynorphin (KNDy) neurons presumably project. Kisspeptin/metastin is pulsatilely released from KNDy neurons, which stimulate the pituitary eventually via pulsatile GnRH secretion. GnRH neural activity may be also supported by kisspeptin independent other neurons. When blocking KISS1R on GnRH neural terminals by KISS1R antagonist, GnRH release is attenuated; however, further studies of KISS1R antagonist would be needed to clarify the effect on kisspeptin independent neural inputs.



Abbreviations

ARC: arcuate nucleus
Dyn: dynorphin
KNDy: kisspeptin/neurokinin B/dynorphin
Kp: kisspeptin/metastin
Kp-a: KISS1R agonists
Kp-ant: KISS1R antagonist
ME: median eminence
NKB: neurokinin B
POA: preoptic area
(+): tonic effect
(–): inhibitory effect

Figure 44. Schematic illustration of hypothalamic GnRH/KNDy neurons and effects of KISS1R agonists/antagonists on pulsatile GnRH/LH release (Matsui and Asami. *Neuroendocrinology* **2014,** *99,* 49-60).

Acute administration of KISS1R agonist stimulates GnRH release, followed by abrupt elevation of plasma LH levels. On the other hand, continuous dosing of KISS1R agonist suppresses GnRH release paradoxically, which inhibits LH secretion. It is potentially because of desensitization of KISS1R on GnRH neurons. Subsequent studies on KISS1R agonist analogues in male rats have also suggested low-level and nonpulsatile leakage of GnRH, which have not been well elucidated only by use of metastin(45-54) with native amino acid sequence.

Compound **56** is the first short-length KISS1R agonist to possess testosterone suppressive activity comparable to that of leuprolide acetate *in vivo* and has led to the elucidation of investigational analogues TAK-683 and TAK-448, both of which have undergone clinical evaluation.

The results in this study suggest that short-length KISS1R agonists can be efficacious in humans, leading to development of novel and effective investigational candidates for the therapy of sex-hormone-dependent diseases such as prostate cancer (**Chapter 4**).

Moreover, in this study biologically stable peptide analogue of endogenous ligand is indicated to be a powerful weapon to elucidate functions of orphan GPCRs with peptide ligands. This study suggests one of the most plausible and general strategy of the drug discovery and development targeting orphan GPCRs.

Chapter 6 Experimental Procedures

6.1. Instruments and Materials.

Peptide synthesis was carried out with an ABI 433A and/or manual shaker using Fmoc chemistry. N^{α}-Fmoc-Rink amide MBHA resin was purchased from Calbiochem-Novabiochem Japan, Ltd (Tokyo, Japan) and Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). PAL resin was purchased from Advanced Chemtech (Louisville, KY, USA). N^{α}-Fmoc-(and side chain) protected amino acids were obtained from Peptide Institute, Inc. (Osaka, Japan), Kokusan Chemical Co., Ltd (Tokyo, Japan), Watanabe Chemical Industries, and Calbiochem-Novabiochem Japan. Side-chain protections were as follows: Arg(Pbf), Asn(Trt), Orn(Mtt), Ser(*t*-Bu), Thr(*t*-Bu), Tyr(*t*-Bu), Trp(Boc). Solvents and other reagents were reagent grade and used without further purification unless otherwise noted. DIPCDI, HOBt, and TFA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Peperidine, PyBOP, PyBrop, and TIS were purchased from Watanabe Chemical Industries. (7-Azabenzotriazol-1-yl oxy)tris(pyrrolidino) phosphonium-hexafluorophosphate (PyAOP) was purchased from PerSeptive Biosystems Inc. (Hamburg, Germany). The substitution of the resins was determined by spectrophotometric analysis (Shimadzu UV-160A UV/vis spectrophotometer) at 290 nm of the dibenzofulvene-piperidine adduct formed upon deprotection of the amino terminal Fmoc group.

The crude peptides were purified to homogeneity by RP-HPLC. HPLC conditions: YMC C18 column (20 × 150 mm), YMC C18 column (20 × 250 mm) or YMC C18 column (30 × 250 mm); solvent gradient, A ,0.1% TFA in water; B, 0.1% TFA in acetonitrile with gradient indicated below; flow rate, 8 mL/min for C18 column (20 ×

150 mm, 20 × 250 mm), 20 mL/min for C18 column (30 × 250 mm); UV detector, 220/254 nm.

The purity of products was characterized by analytical HPLC. Reversed phase HPLC analyses were performed with a Shimadzu gradient system using MERCK Chromolith® FastGradient RP-18 endcapped column (2.0 × 50 mm). Prior to injection, 1 mM DMSO solution of each peptide was diluted with water to give a 100 μM solution; 10 μL of each solution was injected into the HPLC system. For reversed phase HPLC analyses, all peptides were eluted with a linear gradient 0–50% acetonitrile in water containing 0.1% TFA at a flow rate 0.5 mL/min and purity was > 90% at the wavelength of 210 or 220 nm. Molecular weights of the peptides were confirmed by MALDI-TOF MS on a Voyager DE-PRO system (Applied Biosystems, Foster City, CA, USA).

6.2. General Procedures for Synthesis of Metastin Analogues.

All peptides were synthesized by standard Fmoc-based solid phase synthetic methods. Obtained crude peptides were purified to homogeneity with preparative HPLC. The purity of each peptide was ascertained by analytical HPLC, and the structure assignment was performed by MALDI-TOF MS. Structures, HPLC retention times, and MALDI-TOF MS data for each peptide are shown in **Tables 15-21**. Determined masses were in full agreement with calculated masses. The general procedure is described below as the synthesis of [Arg(Me)^{53}]metastin(45-54) compound **8** and [azaGly^{51}]metastin(45-54) compound **36**.

As for compound **8**, in 20 mL of dry DMF, 360 mg of 60% NaH in oil was dissolved and 10 mL of dry DMF solution of 2793 mg of *N*,*N*′-Bis-Boc-1-guanylpyrazole was

added to the solution at 0 °C. The mixture was stirred for 10 minutes. Then, 748 μL of methyl iodide was added to the mixture, followed by stirring at room temperature for 24 hours. After the solvent was removed by distillation, the residue was dissolved in AcOEt, and washed with satd. $NaHCO_3$ aq. solution and then with satd. NaCl aq. solution. After drying over Na_2SO_4, the solvent was concentrated. Purification by flash column chromatography gave 2.96 g of *N*-methyl-*N*,*N'*-bis-Boc-1-guanylpyrazole (yield 91%). Using 480 mg of Fmoc-Phe-Rink Amide MBHA resin, which was prepared by introducing Fmoc-Phe into Rink Amide MBHA resin commercially available, the peptide chain was extended on ABI 433A to give 1080 mg of Boc-Tyr(*t*-Bu)Asn(Trt)Trp(Boc)Asn(Trt)Ser(*t*-Bu)PheGlyLeuOrn(Mtt)Phe-Rink Amide MBHA resin. To 540 mg of the peptide, 10 mL of TFA/TIS/DCM (1/5/94) was added and the mixture was shaken for 50 minutes. The resin was washed and then dried. After 2 mL of DMF, 49 mg of *N*-methyl-*N*,*N'*-bis-Boc-1-guanylpyrazole and 87 μL of DIEA were added to 2/5 volume of the resin, the mixture was shaken for 15 hours to give 220 mg of Boc-Tyr(*t*-Bu)Asn(Trt)Trp(Boc)Asn(Trt)Ser(*t*-Bu)PheGlyLeuArg(Boc_2,Me)Phe-Rink Amide MBHA resin. To 50 mg of the peptide, 1 mL of TFA/thioanisole/*m*-cresol/TIS/EDT (85/5/5/2.5/2.5) was added and the mixture was stirred for 2 hours. Diethyl ether was added to the reaction solution, the resulting precipitate was centrifuged and the supernatant was removed. This procedure was repeated for washing. The residue was extracted with an aqueous acetic acid solution and the extract was filtered to remove the resin. Then, linear density gradient elution (30 minutes) was performed with eluants A/B: 74/26-64/36 using: 0.1% TFA in water and eluant B: 0.1% TFA-containing acetonitrile on preparative HPLC using YMC D-ODS-5-ST S-5 120A column (20 × 150 mm). The fractions containing the product

were collected and lyophilized to give 10.5 mg of white powders; mass spectrum: $(M+H)^+$ 1316.84 (Calcd. 1316.65). Elution time on RP-HPLC: 9.12 min.

Elution conditions: MERCK Chromolith® FastGradient RP-18 endcapped column (2.0 × 50 mm), linear density gradient elution with eluents A/B = 100/0-50/50 (10 min), using 0.1% TFA in water as eluent A and 0.1% TFA-containing acetonitrile as eluant B; flow rate: 0.5 mL/min.

With regard to compound **36**, using 321 mg of Fmoc-Phe-PAL resin, which was prepared by the introduction of Fmoc-Phe into PAL resin, the peptide chain was elongated and 80 mg of Fmoc-LeuArg(Pbf)Phe-PAL resin thus extended was subjected to Fmoc deprotection. After 2 mL of THF and 16 mg of CDI were added, the mixture was shaken for 2 hours. Then 6 μL of hydrazine monohydrate was added to the mixture. The mixture was shaken for an hour and the resin was then washed. After 39 mg of Fmoc-Phe, 93 mg of PyBrop, 27 mg of HOAt and 105 μL of DIEA were added to the mixture, followed by shaking for 2 hours. The resin was washed and the peptide chain was extended to give Boc-Tyr(*t*-Bu)Asn(Trt)Trp(Boc)Asn(Trt)Ser(*t*-Bu)Phe azaGlyLeuArg(Pbf)Phe-PAL resin. To 25 mg of the resin, 1 mL of TFA/thioanisole/*m*-cresol/TIS/EDT (85/5/5/2.5/2.5) was added, and the mixture was shaken for 2 hours. Diethyl ether was added to the reaction solution, the resulting precipitate was centrifuged and the supernatant was removed. This procedure was repeated for washing. The residue was extracted with an aqueous acetic acid solution and the extract was filtered to remove the resin. Linear density gradient elution (30 minutes) was performed with eluents A/B: 74/26-64/36 using: 0.1% TFA in water and eluent B: 0.1% TFA-containing acetonitrile on preparative HPLC using YMC

D-ODS-5-ST S-5 120A column (200 × 150 mm). The fractions containing the product were collected and lyophilized to give 5.5 mg of white powders; mass spectrum: $(M+H)^+$ 1303.79 (Calcd. 1303.63). Elution time on RP-HPLC: 7.91 min.

Elution conditions: MERCK Chromolith® FastGradient RP-18 endcapped column (2.0 × 50 mm), linear density gradient elution with eluents A/B = 100/0-50/50 (10 min), using 0.1% TFA in water as eluent A and 0.1% TFA-containing acetonitrile as eluent B; flow rate: 0.5 mL/min.

6.3. Calcium Mobilization Assay.

Intracellular calcium flux was measured as the increase in fluorescence emitted by the calcium-binding fluorophore using the fluo-4 NW calcium kit (Life Technologies Japan, Tokyo, Japan). Human KISS1R-transfected CHO cells were seeded into 96-well cell plates (Corning International, Tokyo, Japan) at 15,000 cells/well and cultured for an 24 h until used in the functional calcium mobilization assays. Wells were then loaded with 100 μL of HANKS/HBSS-4 mM probenecid-0.1% BSA sample buffer containing Fluo-4 Direct calcium assay reagent solution (Life Technologies Japan, Tokyo, Japan) for 30 minutes at 37 °C (5% CO_2) and 30 minutes at room temperature. Compounds were dissolved in DMSO as 10 μM stock solutions and diluted appropriately with the sample buffer in 96-well sample plates (V-Bottom plate type 3363, Corning International, Tokyo, Japan**)**. The CellLux-Cellular Fluorescence Workstation (PerkinElmer, MA, USA) was programmed to transfer 50 μL from each well of the sample plate to each well of the cell plate and to record fluorescence for 2 min, in 2 s intervals. Peak fluorescence counts were used to determine agonist activity. The instrument software normalized the fluorescent reading to give equivalent initial

readings at time zero.

6.4. Mouse Serum Stability Test.

Mouse blood was supplied in-house and a serum pool was prepared. Mouse serum was obtained after incubation at 37 °C for 10 min followed by centrifugation at 13,000 rpm at room temperature for 10 min. Aliquots were stored frozen at –80 °C and thawed just before use. Three independent experiments were carried out for each analogue. In each experiment, a 45 μL mouse serum aliquot was spiked with 5 μL of 1 mM aq. solution of a given metastin analogue. The mixtures were incubated at 37 °C for up to 4 h, depending on the experiment. Metastin analogue/serum aliquots of 50 μL were taken at time 0 and at various time intervals, and quenched immediately by boiling for 3 min. The amount of peptide in serum extracts was determined from the area under HPLC peaks. The percent of a given metastin analogue recovered from serum after incubation at 37 °C (stability index) was calculated relative to the amount of peptide extracted from serum at time 0 taken as 100%.

6.5. Assay for Cell Growth Inhibition Activity in hKISS1R-expressed CHO Cells.

Human KISS1R-expressing CHO cells (hKISS1R) were cultured in DMEM supplemented with 10% dialyzed FBS (10% dFBS/DMEM), which was used for the following assay. hKISS1R cells were suspended in 10% dFBS/DMEM at 10,000 cells/mL. The cells were plated in a 96-well plate at 100 μL/well (1,000 cells/well), followed by culturing overnight at 37 °C in a 5% CO_2 incubator. On the following day, the medium was removed and 90 μL of 10% dFBS/DMEM supplemented with 0.5% BSA (0.5% BSA/10% dFBS/DMEM) was added. Subsequently, 10 μL of a solution of metastin or a metastin derivative in 0.5% BSA/10% dFBS/DMEM was added to each

well, followed by culturing at 37 °C in a 5% CO_2 incubator for 3 days. After 10 μL of Cell Counting Kit-8 solution (Dojin Chemical Laboratory, Kumamoto, Japan) was added to each well, incubation was performed at 37°C in a 5% CO_2 incubator for 4 h and absorbance was measured at 450 nm.

6.6. Induction of Ovulation in Immature Rats Using Metastin Derivatives.

eCG and hCG (serotropin and gonatropin, respectively, Asuka Pharmaceutical Co. Ltd., Tokyo, Japan) were dissolved in saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) at 100 IU/mL. Using a 1 mL tuberculin syringe with a 26-gauge needle (Terumo Co. Ltd, Tokyo, Japan), eCG was subcutaneously injected into the dorsal area of 23-day-old female Wistar rats between 9:00 and 10:00 AM (Charles River Japan, Inc., Kanagawa, Japan) at a dose of 10 IU/animal. Two days after the eCG injection, test agents were dissolved in saline and injected dorsally into 5 rats/group (control = 6 rats) as follows: Group A: 10 IU hCG (0.1 mL); Group B: 100 nmol/kg human metastin; Group C: 100 nmol/kg compound **56**; Group D: 30 nmol/kg compound **56**; Group E: Saline.

Following administration of the investigational agents, each animal was sacrificed by decapitation after 24–25 h to recover blood, bilateral oviducts, and uterus. Blood was collected into tubes containing aprotinin solution (Trasylol; Bayer HealthCare Pharmaceuticals, Leverkusen, Germany) and EDTA to prevent clotting, and centrifuged at 2000 G for 25 min to obtain plasma.

The number of oöcytes was counted as previously described.[5] Where retained oöcytes in the oviducal ampulla were confirmed by stereomicroscopy, the ampulla was

punctured with a 27-gauge syringe needle (Terumo) to retrieve the oöcytes. After granulosa cells surrounding the oöcytes were removed by trypsin treatment, the number of oöcytes was counted. Where the retained oöcytes in the oviductal ampulla were not confirmed by stereomicroscopy, a 27-gauge needle with a polished tip was inserted into the tubal ostium and more than 400 μL of saline was flushed into the oviduct and uterus and the presence or absence of oöcytes in the effluent was determined.

6.7. Evaluation of the Effects of Metastin Peptide Derivatives on Blood Testosterone Levels in Mature Male Rats.

A metastin peptide derivative was dissolved in distilled water (Otsuka Joryusui K.K.) and loaded into 5 ALZET® osmotic pumps (2 mM in 0.2 mL, release rate: 0.001 mL/h, Model 2001, DURECT Corporation, Cupertino, CA, USA). Each filled ALZET® pump was implanted subcutaneously into the dorsal region of 9-week old male CD(SD)IGS or Copenhagen rats (5 rats/group; Charles River Japan, Inc.) under ether anesthesia. For negative controls, distilled water (Otsuka Pharmaceutical Co., Ltd) was loaded in 5 ALZET® osmotic pumps, which were similarly implanted. These pump-implanted rats were fed for 6 days under normal feeding conditions. After weighing, animals were decapitated to collect blood in tubes containing aprotinin and EDTA 2Na to recover plasma as described above. From the obtained plasma, 0.05 mL was applied to RIA (DPC. Total Testosterone Kit, Siemens Healthcare Diagnostics Inc., Deerfield, IL) to measure the plasma testosterone level of each rat. The value below the limit of detection (0.04 ng/mL) in RIA was treated as 0.04 and results were expressed as percent of control value.

Table 15. Chemical data for decapeptide metastin analogues.

H-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-AA^{53}-Phe-NH_2

compound	AA^{53}	RP-HPLC[a]		mass (Da)[b]	
		t_{ret} (min)	purity (%)	theoretical	calculated
metastin(45-54)	Arg				
1	Ala	8.83	96.36	1239.56	1239.86
2	Leu	9.84	93.10	1281.60	1281.93
3	Nle	9.61	96.67	1281.60	1281.77
4	Cit	8.40	96.16	1325.60	1325.68
5	Lys	8.25	95.67	1274.63	1273.95
6	D-Arg	7.89	99.73	1302.64	1302.85
7	Orn	8.68	100.00	1260.62	1260.49
8	Arg(Me)	9.12	98.37	1316.65	1316.84
9	Arg(Me_2)asy	8.79	95.81	1330.67	1330.91
10	Arg(Me_2)sy	8.21	100.00	1330.67	1330.66

[a] Retention times and purities of peptides were characterized by reversed phase HPLC analyses performed with a Shimadzu gradient system using MERCK Chromolith® FastGradient RP-18 endcapped (4.6 × 50 mm) at a wavelength of 220 nm. Peptides were eluted with a linear gradient 0-50% acetonitrile in water containing 0.1% TFA over 10 min (0.5 mL/min). [b] [$M+H^+$] values of molecular weights of peptides were determined by Voyager MALDI-TOF mass spectrometer. Regarding **1**, **2**, **3**, and **4**, [$M+Na^+$] values were detected.

Table 16. Chemical data for decapeptide metastin analogues.

H-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-AA^{53}-Phe-NH_2

compound	AA^{53}	RP-HPLC[a]		mass (Da)[b]	
		t_{ret} (min)	purity (%)	theoretical	calculated
11	Arg(Et)	8.38	96.86	1330.67	1330.57
12	Arg(Pr)	8.40	100.00	1344.69	1344.66
13	Har	8.75	93.98	1316.65	1316.91
14	Har(Me)	8.61	94.24	1330.67	1330.41
15	Nar	8.34	90.22	1288.62	1288.40
16	Nar(Me)	8.71	100.00	1302.64	1302.64
17	$Lys(Me)_2$	8.23	95.95	1324.65	1324.85
18	Dbu	8.46	92.74	1246.60	1246.33
19	Dpr	8.34	94.84	1254.57	1254.86
20	Dpr(Gly)	8.52	100.00	1289.61	1289.34
21	Dpr(AmGly)	8.36	100.00	1331.63	1331.15

[a] Retention times and purities of peptides were characterized by reversed phase HPLC analyses performed with a Shimadzu gradient system using MERCK Chromolith® FastGradient RP-18 endcapped (4.6 × 50 mm) at a wavelength of 220 nm. Peptides were eluted with a linear gradient 0-50% acetonitrile in water containing 0.1% TFA over 10 min (0.5 mL/min). [b] $[M+H^+]$ values of molecular weights of peptides were determined by Voyager MALDI-TOF mass spectrometer. Regarding **17** and **19**, $[M+Na^+]$ values were detected.

Table 17. Chemical data for decapeptide metastin analogues.

H-AA^{45}-Asn-AA^{47}-Asn-Ser-AA$^{50\text{-}51}$-Leu-AA^{53}-Phe-NH_2

compound	AA^{45}	AA^{47}	AA$^{50\text{-}51}$	AA^{53}	RP-HPLC[a]		mass (Da)[b]	
					t_{ret} (min)	purity (%)	theoretical	calculated
metastin(45-54)	Tyr	Trp	Phe-Gly	Arg				
22	D-Tyr	Trp	Phe-Gly	Arg(Me)	7.96	95.48	1316.65	1316.97
23	D-Tyr	Trp	Phe-ψ(CSNH)-Gly	Arg(Me)	9.98	100.00	1332.63	1332.58
24	D-Tyr	Trp	Phe-ψ(CH_2NH)-Gly	Arg(Me)	7.76	92.20	1302.67	1302.90
25	D-Tyr	Trp	Phe-ψ(NHCO)-Gly	Arg(Me)	8.07	90.21	1316.65	1316.51
26	D-Tyr	Trp	Phe-azaGly	Arg(Me)	8.10	90.15	1317.65	1317.61
27	D-Tyr	Ser	Phe-azaGly	Arg(Me)	7.10	100.00	1218.67	1218.32
28	D-Tyr	Thr	Phe-azaGly	Arg(Me)	7.45	100.00	1232.75	1232.62

[a] Retention times and purities of peptides were characterized by reversed phase HPLC analyses performed with a Shimadzu gradient system using MERCK Chromolith® FastGradient RP-18 endcapped (4.6 × 50 mm) at a wavelength of 220 nm. Peptides were eluted with a linear gradient 0-50% acetonitrile in water containing 0.1% TFA over 10 min (0.5 mL/min). [b] Molecular weights of peptides were determined by Voyager MALDI-TOF mass spectrometer.

Table 18. Chemical data for decapeptide metastin analogues.

H-AA^{45}-Asn-AA^{47}-Asn-Ser-AA$^{50\text{-}51}$-Leu-AA^{53}-Phe-NH_2

compound	AA^{45}	AA^{47}	AA$^{50\text{-}51}$	AA^{53}	RP-HPLC[a]		mass (Da)[b]	
					t_{ret} (min)	purity (%)	theoretical	calculated
29	D-Tyr	Ile	Phe-azaGly	Arg(Me)	7.52	100.00	1244.70	1244.65
30	D-Tyr	Cha	Phe-azaGly	Arg(Me)	8.03	91.95	1284.65	1284.46
31	D-Tyr	Pya(3)	Phe-azaGly	Arg(Me)	7.32	100.00	1279.51	1279.63
32	D-Tyr	Pya(4)	Phe-azaGly	Arg(Me)	6.97	99.59	1279.35	1279.63
33	D-Tyr	Nal(2)	Phe-azaGly	Arg(Me)	8.31	100.00	1328.46	1328.65
34	D-Tyr	D-Trp	Phe-azaGly	Arg(Me)	7.85	95.14	1317.47	1317.45
35	D-Tyr	D-Pya(4)	Phe-azaGly	Arg(Me)	6.97	97.62	1279.71	1279.63

[a] Retention times and purities of peptides were characterized by reversed phase HPLC analyses performed with a Shimadzu gradient system using MERCK Chromolith® FastGradient RP-18 endcapped (4.6 × 50 mm) at a wavelength of 220 nm. Peptides were eluted with a linear gradient 0-50% acetonitrile in water containing 0.1% TFA over 10 min (0.5 mL/min). [b] Molecular weights of peptides were determined by Voyager MALDI-TOF mass spectrometer.

Table 19. Chemical data for decapeptide metastin analogues.

H-AA^{45}-AA^{46}-AA^{47}-Asn-Ser-Phe-AA^{51}-Leu-AA^{53}-Phe-NH_2

compound	AA^{45}	AA^{46}	AA^{47}	AA^{51}	AA^{53}	HPLC[a]		mass (Da)[b]	
						t_{ret} (min)	purity (%)	calculated	theoretical
Metastin(45-54)	Tyr	Asn	Trp	Gly	Arg				
36	Tyr	Asn	Trp	azaGly	Arg	9.03	100.00	1303.79	1303.63
37	D-Tyr	D-Asn	Trp	azaGly	Arg(Me)	8.44	98.75	1317.82	1317.65
38	D-Tyr	D-Ser	Trp	azaGly	Arg(Me)	8.66	100.00	1290.74	1290.64
39	D-Tyr	D-His	Trp	azaGly	Arg(Me)	8.99	100.00	1340.69	1340.67
40	D-Tyr	D-Asn	Phe	azaGly	Arg(Me)	7.82	88.49	1278.84	1278.42
41	D-Tyr	D-Asn	Cha	azaGly	Arg(Me)	8.29	98.47	1284.75	1284.46

[a] Retention times and purities of peptides were characterized by HPLC analyses performed with a Gilson gradient system using MERCK Chromolith® column (4.6 × 50 mm, 2 µm particle size). Peptides were eluted with a linear gradient 0-50% acetonitrile in water containing 0.1% TFA over 25 min. [b] Molecular weights of peptides were determined by Voyager MALDI-TOF mass spectrometer.

Table 20. Chemical data for decapeptide metastin analogues.

H-AA^{45}-AA^{46}-AA^{47}-Asn-Ser-Phe-AA^{51}-Leu-AA^{53}-Phe-NH_2

compound	AA^{45}	AA^{46}	AA^{47}	AA^{51}	AA^{53}	HPLC[a]		mass (Da)[b]	
						t_{ret} (min)	purity (%)	calculated	theoretical
42	D-Tyr	D-Asn	Thr	azaGly	Arg(Me)	7.08	93.32	1232.57	1232.62
43	D-Tyr	D-Asn	Pya(4)	azaGly	Arg(Me)	9.78	100.00	1279.51	1279.63
44	D-Tyr	D-Asn	D-Trp	azaGly	Arg(Me)	8.13	98.76	1317.43	1317.65
45	D-Ala	Asn	Trp	azaGly	Arg(Me)	9.42	100.00	1225.55	1225.62
46	D-Pya(3)	Asn	Trp	azaGly	Arg(Me)	12.15	100.00	1302.58	1302.65
47	D-Pya(3)	D-Asn	Cha	azaGly	Arg(Me)	8.10	99.64	1269.83	1269.69
48	Tyr	D-Asn	Pya(4)	azaGly	Arg(Me)	7.35	94.88	1279.33	1279.63

[a] Retention times and purities of peptides were characterized by HPLC analyses performed with a Gilson gradient system using MERCK Chromolith® column (4.6 × 50 mm, 2 µm particle size). Peptides were eluted with a linear gradient 0-50% acetonitrile in water containing 0.1% TFA over 25 min. [b] Molecular weights of peptides were determined by Voyager MALDI-TOF mass spectrometer.

Table 21. Chemical data for nonapeptide metastin analogues.

H-AA[46]-AA[47]-Asn-Ser-Phe-azaGly-Leu-Arg(Me)-Phe-NH_2

			HPLC[a]		mass (Da)[b]	
compound	AA[46]	AA[47]	t_{ret} (min)	purity (%)	calculated	theoretical
49	D-Tyr	Trp	8.34	99.26	1203.22	1203.61
50	D-Tyr	Ser	7.36	98.38	1104.60	1104.56
51	D-Tyr	Thr	7.47	100.00	1118.75	1118.57
52	D-Tyr	Ile	8.71	97.59	1130.77	1130.61
53	D-Tyr	Phe	8.24	89.96	1164.28	1164.31
54	D-Tyr	Nal(2)	8.88	100.00	1214.43	1214.61
55	D-Tyr	D-Trp	8.85	98.67	1203.24	1203.61
56	D-Tyr	D-Pya(4)	7.08	96.74	1165.28	1165.59
57	D-Pya(3)	Phe	7.82	100.00	1149.60	1149.60
58	D-Pya(3)	Trp	7.76	86.80	1188.43	1188.34

[a] Retention times and purities of peptides were characterized by HPLC analyses performed with a Gilson gradient system using MERCK Chromolith® column (4.6 × 50 mm, 2 µm particle size). Peptides were eluted with a linear gradient 0-50% acetonitrile in water containing 0.1% TFA over 25 min. [b] Molecular weights of peptides were determined by Voyager MALDI-TOF mass spectrometer.

Chapter 7 References

1. Rask-Andersen, M.; Masuram, S.; Schiöth, H. B. The druggable genome: Evaluation of drug targets in clinical trials suggests major shifts in molecular class and indication. *Annu. Rev. Pharmacol. Toxicol.* **2014***, 54*, 9.
2. Civelli, O.; Reinscheid, R. K.; Zhang, Y.; Wang, Z.; Fredriksson, R.; Schiöth, H.B. G protein-coupled receptor deorphanizations.*Annu. Rev. Pharmacol. Toxicol.* **2013**, *53*, 127.
3. Ohtaki, T.; Shintani, Y.; Honda, S.; Matsumoto, H.; Hori, A.; Kanehashi, K.; Terao, Y.; Kumano, S.; Takatsu, Y.; Masuda, Y.; Ishibashi, Y.; Watanabe, T.; Asada, M.; Yamada, T.; Suenaga, M.; Kitada, C.; Usuki, S.; Kurokawa, T.; Onda, H.; Nishimura, O.; Fujino, M. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature* **2001**, *411*, 613.
4. Kotani, M.; Detheux, M.; Vandenbogaerde, A.; Communi, D.; Vanderwinden, J. M.; De Poul, E.; Bre´ zillon, S.; Tyldesley, R.; Suarez-Huerta, N.; Vandeput, F.; Blanpain, C.; Schiffmann, S. N.; Vassart, G.; Parmentier, M. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J. Biol. Chem.* **2001**, *276*, 34631.
5. Muir, A. I.; Chamberlain, L.; Elshourbagy, N.A.; Michalovich, D.; Moore, D. J.; Calamari, A.; Szekeres, P. G.; Sarau, H. M.; Chambers, J. K.; Murdock, P.; Steplewski, K.; Shabon, U.; Miller, J. E.; Middleton, S. E.; Darker, J. G.; Larminie, C. G. C.; Wilson, S.; Bergsma, D. J.; Emson, P.; Faull, R.; Philpott, K. L.; Harrison, D. C. AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. *J. Biol. Chem.* **2001**, *276*, 28969.
6. Lee, J. H.; Miele, M. E.; Hicks, D. J.; Phillips, K. K.; Trent, J. M.; Weissman, B. E.;

Welch, D. R. J. KiSS-1, a Novel Human Malignant Melanoma Metastasis-Suppressor Gene. *Natl. Cancer Inst.* **1996**, *88*, 1731.

7. Lee, J. H.; Welch, D. R. Suppression of Metastasis in Human Breast Carcinoma MDA-MB-435 Cells after Transfection with the Metastasis Suppressor Gene, KiSS-1. *Cancer Res.* **1997**, *57*, 2384.
8. Lee, J. H.; Welch, D. R. Identification of highly expressed genes in metastasis-suppressed chromosome 6/human malignant melanoma hybrid cells using subtractive hybridization and differential display. *Int. J. Cancer* **1997**, *71*, 1035.
9. Matsui, H.; Takatsu, Y.; Kumano, S.; Matsumoto, H.; Ohtaki, T. Peripheral administration of metastin induces marked gonadotropin release and ovulation in the rat. *Biochem. Biophys. Res. Commun.* **2004**, *320*, 383.
10. Gottsch, M. L.; Cunningham, M. J.; Smith, J. T.; Popa, S. M.; Acohido, B. V.; Crowley, W. F.; Seminara, S.; Clifton, D. K.; Steiner, R. A. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology* **2004**, *145*, 4073.
11. Dhillo, W. S.; Chaudhri, O. B.; Patterson, M.; Thompson, E. L.; Murphy; K. G.; Badman, M. K.; McGowan, B. M.; Amber, V.; Patel, S.; Ghatei, M. A.; Bloom, S. R. Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. *Clin. Endocrinol. Metab.* **2005**, *90*, 6609.
12. Messager, S.; Chatzidaki, E. E.; Ma, D.; Hendrick, A. G.; Zahn, D.; Dixon, J.; Thresher, R. R.; Malinge, I.; Lomet, D.; Carlton, M. B.; Colledge, W. H.; Caraty, A.; Aparicio, S. A. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc Natl. Acad. Sci. U. S. A.* **2005**, *102*, 1761-1766.

13. de Roux, N.; Genin, E.; Carel, J. C.; Matsuda, F.; Chaussain, J. L.; Milgrom, E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc. Natl. Acad. Sci. USA.* **2003**, *100*, 10972.
14. Seminara, S. B.; Messager, S.; Chatzidaki, E. E.; Thresher, R. R, Acierno, J. S. Jr; Shagoury, J. K.; Boabbas, Y.; Kuohung, W.; Schwinof, K. M.; Hendrick A. G.; Zahn, D.; Dixon, J.; Kaiser, U. B.; Slaugenhaupt, S. A.; Gusella, J. F.; O'Rahilly, S.; Carlton, M,B.; Crowley, W. F. Jr; Aparicio, S. A.; Colledge W. H. The GPR54 gene as a regulator of puberty. *N. Engl. J. Med.* **2003**, *349*, 1614.
15. Funes, S.; Hedrick, J. A.; Vassileva, G.; Markowitz, L.; Abbondanzo, S.; Golovko, A.; Yang, S.; Monsma, F. J.; Gustafson, E. L. The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. *Biochem. Biophys. Res. Commun.* **2003**, *312*, 1357.
16. Tomita, K.; Oishi, S.; Ohno, H.; Peiper, S. C.; Fujii, N. Development of novel G protein-coupled receptor 54 agonists with resistance to degradation by matrix metalloproteinase. *J. Med. Chem.* **2008**, *51*, 7645.
17. Stroud, R. M. A family of protein-cutting proteins. *Sci. Am.* **1974**, *231*, 74.
18. Kraut, J. Serine Proteases: Structure and Mechanism of Catalysis. *Annu. Rev. Biochem.* **1977**, *46*, 331.
19. Steitz, T.; Shulman, R. Crystallographic and NMR Studies of the Serine Proteases. *Annu. Rev. Biophys. Bioeng.* **1982**, *11*, 419.
20. Curtis, A. E.; Cooke, J. H.; Baxter J. E.; Parkinson, J. R.; Bataveljic, A.; Ghatei, M. A.; Bloom, S. R.; Murphy, K. G. A kisspeptin-10 analog with greater in vivo bioactivity than kisspeptin-10. *Am. J. Physiol. Endocrinol. Metab.* **2010**, *298*, E296.
21. Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Gee, K.; Yamamura, H. I.; Galligan, J. J.; Burks, T. F. Bis-penicillamine enkephalins possess highly improved specificity

toward δ opioid receptors. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5871.

22. Dooley, C. T.; Chung, N. N.; Wilkes, B. C.; Schiller, P. W.; Bidlack, J. M.; Pasternak, G. W.; Houghten, R. N. An all D-amino acid opioid peptide with central analgesic activity from a combinatorial library. *Science* **1994**, *266*, 2019
23. Witkowska, E.; Orłowska, A.; Sagan, B.; Smoluch, M.; Izdebski, J. Tryptic hydrolysis of hGH-RH(1-29)-NH_2 analogues containing Lys or Orn in positions 12 and 21 J. *Pept. Sci.* **2001**, *7*, 166.
24. Izdebski, J.; Witkowska, E.; Kunce, D.; Orłowska, A.; Baranowska, B.; Radzikowska, M.; Smoluch, M. New potent hGH-RH analogues with increased resistance to enzymatic degradation. *J. Pept. Sci.* **2002**, *8*, 289.
25. Kim, K. S.; Qian, L. Improved method for the preparation of guanidines. *Tetrahedron Lett.* **1993**, *34*, 7677.
26. Paik, W. K.; Kim, S. Protein methylase I. Purification and properties of the enzyme. *J. Biol. Chem.* **1968**, *243*, 2108.
27. (a) Spatola, A. F. In Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins; Weinstein, B., Ed.; Marcel Dekker: New York, **1983**; pp 267-357. (b) Sherman, D. B.; Spatola, A. F. Compatibility of thioamides with reverse turn features: synthesis and conformational analysis of two model cyclic pseudopeptides containing thioamides as backbone modifications. *J. Am. Chem. Soc.* **1990**, *112*, 433. (c) Hirschmann, R. Medicinal Chemistry in the Golden Age of Biology: Lessons from Steroid and Peptide Research. *Angew Chem. Int. Ed. Engl.* **1991**, *30*, 1278. (d) Gante, J. Peptidomimetics—Tailored Enzyme Inhibitors. *Angew Chem. Int. Ed. Engl.* **1994**, *33*, 1699.
28. (a) Michel, A. G.; Ameziane-Hassani, C.; Boulay, G. Étude structurale de la liaison thioamide: Synthèse et conformation de dérivés de la thioalanine et de la thioglycine.

Can. J. Chem. **1989**, *67*, 1312. (b) Mock, W. L.; Chen, J. T.; Tsang, J. W. Hydrolysis of a thiopeptide by cadmium carboxypeptidase A. *Biochem. Biophys. Res. Commun.* **1981**, *102*, 389.

29. Clausen, K.; Spatola, A. F.; Lemieux, C.; Schiller, P. W.; Lawesson, S.-O. Evidence of a peptide backbone contribution toward selective receptor recognition for leucine enkephalin thioamide analogs. *Biochem. Biophys. Res. Commun.* **1984**, *120*, 305.
30. Meyer, J.-P.; Davis, P.; Lee, K. B.; Porreca, F.; Yamamura, H. I.; Hruby, V. J. Synthesis Using a Fmoc-Based Strategy and Biological Activities of Some Reduced Peptide Bond Pseudopeptide Analogs of Dynorphin A. *J. Med. Chem.* **1995**, *38*, 3462.
31. Fehrentz, J.-A.; Castro, B. An Efficient Synthesis of Optically Active α-(t-Butoxycarbonylamino) Aldehydes from α-Amino Acids. *Synthesis* **1983**, 676.
32. Sasaki, Y.; Coy, D. H. Solid Phase Synthesis of Peptides Containing the CH_2NH Peptide Bond Isostere. *Peptides* **1987**, *8*, 119.
33. Pessi, A.; Pinori, M.; Verdini, A. S.; Viscomi, G. C. Solid phase synthesis of retro-inverso peptides. Synthesis of the partially modified retro-inverso analog of substance P Glp6,gPhe8,mGly9rSP6-11. *Farmaco* **1983**, 38, 360.
34. Lee, H.-J.; Ahn, I.-A.; Ro, S.; Choi, K.-H.; Choi, Y.-S.; Lee, K.-B. J. Role of azaamino acid residue in beta-turn formation and stability in designed peptide. *Pept. Res.* **2000**, *56*, 35.
35. Andre´, F.; Vicherat, A.; Boussard, G.; Aubry, A.; Marraud, M. Aza-peptides. III. Experimental structural analysis of aza-alanine and aza-asparagine-containing peptides. *J. Pept. Res.* **1997**, *50*, 372.
36. Zouikri, M.; Vicherat, A.; Aubry, A.; Marraud, M.; Boussard, G. Azaproline as a β-turn-inducer residue opposed to proline. *J. Pept. Res.* **1998**, *52*, 19.

37. Lee, H.-J.; Choi, K.-H.; Ahn, I.-A.; Ro, S.; Jang, H. G.; Choi, Y.-S.; Lee, K.-B. The β-turn preferential solution conformation of a tetrapeptide containing an azaamino acid residue. *J. Mol. Struct.* **2001**, *569*, 43.

38. Lecoq, A.; Boussard, G.; Marraud, M. Solid phase synthesis of a cyclic peptide derived from a curaremimetic toxin. *Tetrahedron Lett.* **1992**, *33*, 5209.

39. Didierjean, C.; Del Duca, V.; Benedetti, E.; Aubry, A.; Zouikri, M.; Marraud, M.; Boussard, G. X-ray structures of aza-proline-containing peptides. *J. Pept. Res.* **1997**, *50*, 451.

40. Andre´, F.; Boussard, G.; Bayeul, D.; Didierjean, C.; Aubry, A.; Marraud, M. Aza-peptides II. X-Ray structures of aza-alanine and aza-asparagine-containing peptides. *J. Pept. Res.* **1997**, *49*, 556.

41. Benatalah, Z.; Aubry, A.; Boussard, G.; Marraud, M. Evidence for a β-turn in an azadipeptide sequence : Synthesis and crystal structure of $Bu^{t}CO$-Pro-AzaAla-$NHPr^{i}$. *Int. J. Pept. Protein Res.* **1991**, *38*, 603.

42. Lecoq, A.; Boussard, G.; Marraud, M.; Aubry, A. Crystal state conformation of three azapeptides containing the Azaproline residue, a β-turn regulator. *Biopolymers* **1993**, *33*, 1051.

43. Thormann, M.; Hofmann, H.-J. *J. Mol. Struct. (THEOCHEM)* Conformational properties of azapeptides. **1999**, *469*, 63.

44. Lee, H.-J.; Song, J.-W.; Choi, Y.-S.; Ro, S.; Yoon, C. The energetically favorable cis peptide bond for the azaglycine-containing peptide: For-AzGly-NH_2 model. *Phys. Chem. Chem. Phys.* **2001**, *3*, 1693.

45. Zhang, W. J.; Berglund, A.; Kao, J. L.-F.; Couty, J. P.; Gershengorn, M. C.; Marshall, G. R. Impact of azaproline on amide cis-trans isomerism: conformational analyses and NMR studies of model peptides including TRH analogues. *J. Am. Chem. Soc.*

2003, *125*, 1221.

46. Lee, H. J.; Song, J. W.; Choi, Y. S.; Park, H. M.; Lee, K. B. A theoretical study of conformational properties of N-methyl azapeptide derivatives. *J. Am. Chem. Soc.* **2002**, *124*, 11881.
47. Lee J. Y.; Moon J.S.; Eu Y.J.; Lee C. W.; Yang S. T.; Lee S. K.; Jung H. H; Kim H. H.; Rhim H.; Seong J. Y. ; Kim J. I. Molecular interaction between kisspeptin decapeptide analogs and a lipid membrane. *Arch. Biochem. Biophys.* **2009**, *485*, 109.
48. Wilcox, P. E. Chymotrypsinogens—chymotrypsins. *Method Enzymol.* **1970**, *19*, 64.
49. Appel, W. Chymotrypsin: molecular and catalytic properties. *Clin. Biochem.* **1986**, *19*, 317
50. Niida, A.; Wang, Z.; Tomita, K.; Oishi, S.; Tamamura, H.; Otaka, A.; Navenot, J. M.; Broach, J. R.; Peiper, S. C.; Fujii, N. Design and synthesis of downsized metastin (45-54) analogs with maintenance of high GPR54 agonistic activity. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 134.
51. Gutiérrez-Pascual E.; Leprince J,; Martínez-Fuentes A. J.; Ségalas-Milazzo I; Pineda R.; Roa J.; Duran-Prado, M.; Guilhaudis, L.; Desperrois, E.; Lebreton, A.; Pinilla, L.; Tonon, M. C.; Malagón, M. M.; Vaudry, H.; Tena-Sempere, M.; Castaño, J. P. In Vivo and in Vitro Structure-Activity Relationships and Structural Conformation of Kisspeptin-10-Related Peptides. *Mol. Pharmacol.* **2009**, *76*, 58.
52. Niedrich, H. Hydrazinverbindungen als Heterobestandteile in Peptiden. XVI. Synthese von 9-Hydrazinoessigsäure-, 9-Azaglycin- und 5-α-Azaasparagin-Oxytocin. *J. Prakt. Chem.* **1972**, *314*, 769.
53. Niedrich, H.; Oehme, C. Hydrazinverbindungen als Heterobestandteile in Peptiden. XV. Synthese von Eledoisin-Octapeptiden mit den Carbazylresten Azaglycin und α-Azaasparagin statt Glycin und Asparagin. *J. Prakt. Chem.* **1972**, *314*, 759.

54. Oehme, P.; Bergmann, J.; Falk, M.; Reich, J. G.; Voigt, W. E.; Niedrich, H.; Pirrwitz, J.; Bersek, C.; Jung, F. Pharmacology of hydrazino carbonic acids, hydrazino peptides and other hydrazine derivatives. Structure-action studies in heterologeous eledoisin octapeptide sequences. *Acta Biol. Med. Ger.* **1972**, *28*, 109.

55. Oehme, P.; Bergmann, J.; Niedrich, H.; Jung, F.; Menzel, G.; Eichstädt, M. Pharmacology of hydrazine-carbonic acids, hydrazine peptides and other hydrazine derivatives. VII. Pharmacologic studies on heterologous eledoisine sequences. *Acta Biol. Med. Ger.* **1970**, *25*, 613.

56. Han, H.; Yoon, J.; Janda, K. D. Investigations of azapeptides as mimetics of Leu-enkephalin. *Biorg. Med. Chem. Lett.* **1998**, *8*, 117.

57. Dutta, A. S.; Gormley, J. J.; Hayward, C. F.; Morley, J. S.; Shaw, J. S.; Stacey, G. J.; Turnbull, M. T. Enkephalin analogues eliciting analgesia after intravenous injection. *Life Sci.* **1977**, *21*, 559.

58. Dutta, A. S.; Gormley, J. J.; Hayward, C. F.; Morley, J. S.; Shaw, J. S.; Stacey, G. J.; Turnbull, M. T. Analgesia following intravenous administration of enkephalin analogues. *Br. J. Pharmacol.* **1977**, *61*, 481.

59. Ho, T. L.; Nestor, J. J.; McCrae, G. I.; Vickery, B. H. Hydrophobic, aza-glycine analogues of luteinizing hormone-releasing hormone. *Int. J. Peptide Protein Res.* **1984**, *24*, 79.

60. Dutta, A. S.; Furr, B. J. A.; Giles, M. B.; Valcaccia, B.; Walpole, A. L. Potent agonist and antagonist analogues of luliberin containing an azaglycine residue in position 10. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 382.

61. Furr, B. J. A.; Valcaccia, B. E.; Hutchinson, F. G. Response of rat mammary and prostate tumours to treatment with a biodegradable slow-release formulation of the LH-RH analogue, ICI 118630. *Brit. J. Cancer* **1983**, *48*, 140.

62. Dutta, A. S.; Furr, B. J. A. Luteinizing Hormone Releasing Hormone (LHRH) Analogues. *Ann. Rep. Med. Chem.* **1985**, *20*, 203.

63. Zhang, W. J.; Berglund, A.; Kao, J. L. F.; Couty, J. P. Gershengorn, M. C.; Marshall, J. R. Impact of azaproline on amide cis-trans isomerism: conformational analyses and NMR studies of model peptides including TRH analogues. *J. Am. Chem. Soc.* **2003**, *125*, 1221.

64. Robinson, N. E.; Robinson, A. B. Molecular clocks. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 944-949.

65. Robinson, A. B.; McKerrow, J. H.; Cary, P. Controlled deamidation of peptides and proteins: an experimental hazard and a possible biological timer. *Proc. Natl. Acad. Sci. U. S. A.* **1970**, *66*, 753-757.

66. Hori, A.; Honda, S.; Asada, M.; Ohtaki, T.; Oda, K.; Watanabe, T.; Shintani, Y.; Yamada, T.; Suenaga, M.; Kitada, C.; Onda, H.; Kurokawa, T.; Nishimura, O.; Fujino, M. Metastin suppresses the motility and growth of CHO cells transfected with its receptor. *Biochem. Biophys. Res. Commun.* **2001**, *286*, 958-963.

67. Seminara, S. B.; Dipietro, M. J.; Ramaswamy, S.; Crowley, W. F., Jr.; Plant, T. M. Continuous human metastin 45-54 infusion desensitizes G protein-coupled receptor 54-induced gonadotropin-releasing hormone release monitored indirectly in the juvenile male Rhesus monkey (Macaca mulatta): a finding with therapeutic implications. *Endocrinology* **2006**, *147*, 2122-2126.

68. Thompson, E. L.; Murphy, K. G.; Patterson, M.; Bewick, G. A.; Stamp, G. W.; Curtis, A. E.; Cooke, J. H.; Jethwa, P. H.; Todd, J. F.; Ghatei, M. A.; Bloom, S. R. Chronic subcutaneous administration of kisspeptin-54 causes testicular degeneration in adult male rats. *Am. J. Physiol. Endocrinol. Metab.* **2006**, *291*, E1074.

69. Leuprolide for prostate cancer. *Med. Lett. Drugs Ther.* **1985**, *27*, 71-72.

70. Matsui, H.; Tanaka, A.; Yokoyama, K.; Takatsu, Y.; Ishikawa, K.; Asami, T.; Nishizawa, N.; Suzuki, A.; Kumano, S.; Terada, M.; Kusaka, M.; Kitada, C.; Ohtaki, T. Chronic administration of the metastin/kisspeptin analog KISS1-305 or the investigational agent TAK-448 suppresses hypothalamic pituitary gonadal function and depletes plasma testosterone in adult male rats. *Endocrinology* **2012**, *153*, 5297-308.

Acknowledgements

The author wishes to thank Drs. Chieko Kitada, Tetsuya Ohtaki and Masahiko Fujino of Takeda Pharmaceutical Company Ltd. for their valuable advice and continuous encouragement during the course of this study.

The author would like to thank Drs. Shigenori Ohkawa, Masahiro Sumino, Kyozo Tsukamoto, Osamu Nishimura, Yukio Fujisawa, Kenji Okonogi, Nobuhiro Suzuki, Masami Kusaka, Tatsuya Watanabe, Yuji Ishihara, Kaneyoshi Kato, Osamu Uchikawa, Fumio Itoh, Akihiro Tasaka, Keiji Kamiyama, Tomoyasu Ishikawa, Yan Zu, Nobuo Cho, Atsuo Baba, Kohji Fukatsu, Tooru Yamano and Mitsuhiro Wakimasu of Takeda Pharmaceutical Company Ltd. for their kind support and continuous encouragement.

The author deeply thanks all the members that have provided me with data and information, in particular, Drs. Naoki Nishizawa, Yoshihiro Ishibashi, Hisanori Matsui, Kimiko Nishibori, Hirokazu Matsumoto, Yasuko Horikoshi, Naoki Tarui, Masaharu Nakayama, Masashi Yamaguchi, and Shin-ichi Matsumoto of Takeda Pharmaceutical Company Ltd.

Thanks are also due to Drs. Yasushi Masuda, Michiko Terada, Yoshihiro Takatsu, Satoshi Kumano, Atsushi Kiba, Atsuko Suzuki, Mika Kinoshita, Yasushi Shintani, Susumu Honda, Yasuko Terao, Takuya Watanabe, Jiro Noguchi, Nobuyo Yoshida, Eri Shiraishi, Hiromi Yoshida, Kotaro Yokoyama, Akira Hori, Mari Asada, Mie Yoshimatsu, Akita Tanaka, Daisuke Nakata, Yoshio Anazawa, Yumiko Akinaga, Tsuneo Masaki, Kaori Ishikawa, Hidenori Kamiguchi, Mikio Shirasaki, Yukihiro Ikeda, Junko Ban,

Shunichiro Tsutsumi, Masahiro Kawase, Satoru Asahi, Takahiro Kondo, Yoshihiko Tagawa, Fumihiro Jinno, Akio Morohashi, Yoji Kuze, Yuu Moriya, Fumio Chatani, Hideo Fukui, Makoto Kamata, Toshio Miyawaki, Kazuhiro Miwa, Katsuhiko Miwa, Kazuhiro Tsuchinaga, Toshitake Kobayashi, Ayumu Niida, Takao Yamada, Masato Suenaga, Shokyo Miki, Mitsuhiro Takeda, Toshihiko Fujitani, Atsushi Inagaki, Midori Echigoya, Yutaka Yamagata, Tomomichi Futo and Hikaru Taira of Takeda Pharmaceutical Company Ltd. for their helpful discussion and support.

The author would like to give special thanks to Professor Tomohiko Ohwada of The University of Tokyo for his critical reading and scientific discussion.

Thanks to all members around me in Takeda Pharmaceutical Company Ltd. for their encouragement.

Finally, I would like to thank my family for their support, assistance and encouragement during the course of the study.