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Serum stability of selected decapeptide agonists of KISS1R using pseudopeptides

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ABSTRACT

Metastin/kisspeptin, a 54-amino acid peptide, is the ligand of the G-protein-coupled receptor KISS1R which plays a key role in pathways that regulate reproduction and cell migration in many endocrine and gonadal tissues. 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 agonistic decapeptide analogs 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 analogs that were highly stable in mouse serum. D-Trp^{47} analog **13** showed not only high metabolic stability but also excellent KISS1R agonistic activity. Other labile peptides may have increased serum stability using amino acid substitution.

Metastin/kisspeptin is the endogenous ligand of an orphan G-protein-coupled receptor GPR54, also known as AXOR12 or OT7T175, and has recently been renamed the KiSS1 receptor (KISS1R).[1–3] Recent studies have suggested that metastin acts as a critical potentiator of gonadotropin-releasing hormone secretion following acute administration in several mammalian species including rats,[4] mice,[5] and human males,[6] but lowers testosterone levels as a consequence of continuous administration.[7] These results strongly suggest that appropriate KISS1R agonists can modulate the hypothalamic-pituitary-gonadal axis and may have the potential to be useful for the prevention or treatment of a number of sex hormone-dependent diseases.

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.[1] However, both metastin and metastin(45–54) are susceptible to enzyme-catalyzed hydrolysis.[8] In our laboratory, 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}.[9] Consequently, an analog possessing Arg(Me) was synthesized and found to be metabolically resistant to trypsin-like proteases.[9] There remains a great demand for more stable analogs with other amino acid replacements at fragile sites to improve their pharmacological properties by maintaining or improving agonistic activity and serum stability. In this report, we provide a rational strategy for the design of decapaptide analogs of KISS1R agonists to improve their stability in murine serum.

All peptides were synthesized by standard *N*-(9-fluorenylmethoxycarbonyl;Fmoc)-based solid phase synthetic methodology except where specifically described. 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 1 and 2. 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 1 and 2.

Metastin(45–54) was rapidly metabolized in mouse plasma (Fig. 1). More than 50% of the peptide was metabolized after a 1-min incubation at 37 °C (data not shown). The metabolites

Abbreviations: Arg(Me), N^{ω}-methylarginine; azaGly, azaglycine; $[Ca^{2+}]_i$, intracellular calcium concentration; Cha, β-cyclohexylalanine; DMF, dimethylformamide; ESI-MS, electrospray ionization mass spectrometry; FLIPR, fluorometric imaging plate reader; Fmoc, 9-fluorenylmethoxycarbonyl; GnRH, gonadotropin-releasing hormone; HPLC, high-performance liquid chromatography; KISSR, kisspeptin receptor; MBHA, 4-methylbenzhydrylamine; MMP-9, metalloproteinase-9; NEP, neutral endopeptidase; Nal(2), β-(2-naphthyl)alanine; Pya(3), β-(3-pyridyl)alanine; Pya(4), β-(4-pyridyl)alanine; T/C, treated/control; TFA, trifluoroacetic acid.

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Table 1
Biological activities of metastin analogs substituted between positions 50 and 51

H-AA^{45}-Asn-Trp-Asn-Ser-AA$^{50-51}$-Leu-AA^{53}-Phe-NH$_2$							
Compound-no.	AA^{45}	AA$^{50-51}$	AA^{53}	Agonist activity[a] EC$_{50}$ (nM)	Binding affinity[c] IC$_{50}$ (nM)		Stability in mouse serum
					Human	Rat	Residual ratio (%)[e]
Metastin(45–54)	Tyr	Phe-Gly	Arg	0.049[b]	0.082[d]	0.11[d]	ND[f]
1	D-Tyr	Phe-Gly	Arg(Me)	0.065	0.11	0.26	18.1
2	D-Tyr	Phe-ψ(CSNH)-Gly	Arg(Me)	0.041	2.8	3.3	35.3
3	D-Tyr	Phe-ψ(CH$_2$NH)-Gly	Arg(Me)	0.62	0.61	1.4	12.1
4	D-Tyr	Phe-ψ(NHCO)-Gly	Arg(Me)	0.95	0.51	2.9	46.8
5	D-Tyr	Phe-azaGly	Arg(Me)	0.050	0.16	0.17	39.5

[a] Agonist activities of all peptide analogs were evaluated in a functional assay of human OT7T175, an intracellular calcium mobilization assay using fluorometric imaging plate reader technology. EC$_{50}$ values of all peptide analogs 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 1 h at 37 °C.
[f] Not determined.

Table 2
Biological activities of decapeptide metastin analogs substituted at position 47

H-AA^{45}-Asn-AA^{47}-Asn-Ser-Phe-AA^{51}-Leu-AA^{53}-Phe-NH$_2$								
Compound-no.	AA^{45}	AA^{47}	AA^{51}	AA^{53}	Agonist activity[a] EC$_{50}$ (nM)	Binding affinity[c] IC$_{50}$ (nM)		Stability in mouse serum
						Human	Rat	Residual ratio (%)[e]
Metastin(45-54)	Tyr	Trp	Gly	Arg	0.049[b]	0.082[d]	0.11[d]	ND[f]
6	D-Tyr	Ser	azaGly	Arg(Me)	0.73	0.49	0.43	50.0
7	D-Tyr	Thr	azaGly	Arg(Me)	0.24	0.86	0.83	62.5
8	D-Tyr	Ile	azaGly	Arg(Me)	0.13	0.43	0.46	ND[f]
9	D-Tyr	Cha	azaGly	Arg(Me)	0.10	0.19	0.24	32.0
10	D-Tyr	Pya(3)	azaGly	Arg(Me)	0.24	0.70	0.89	50.6
11	D-Tyr	Pya(4)	azaGly	Arg(Me)	0.11	0.30	0.28	30.7
12	D-Tyr	Nal(2)	azaGly	Arg(Me)	0.098	0.13	0.20	43.6
13	D-Tyr	D-Trp	azaGly	Arg(Me)	0.072	0.19	0.22	56.4
14	D-Tyr	D-Pya(4)	azaGly	Arg(Me)	0.59	0.28	0.28	ND[f]

[a] Agonist activities of all peptide analogs were evaluated in a functional assay of human OT7T175, an intracellular calcium mobilization assay using fluorometric imaging plate reader technology. EC$_{50}$ values of all peptide analogs 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 1 h at 37 °C.
[f] Not determined.

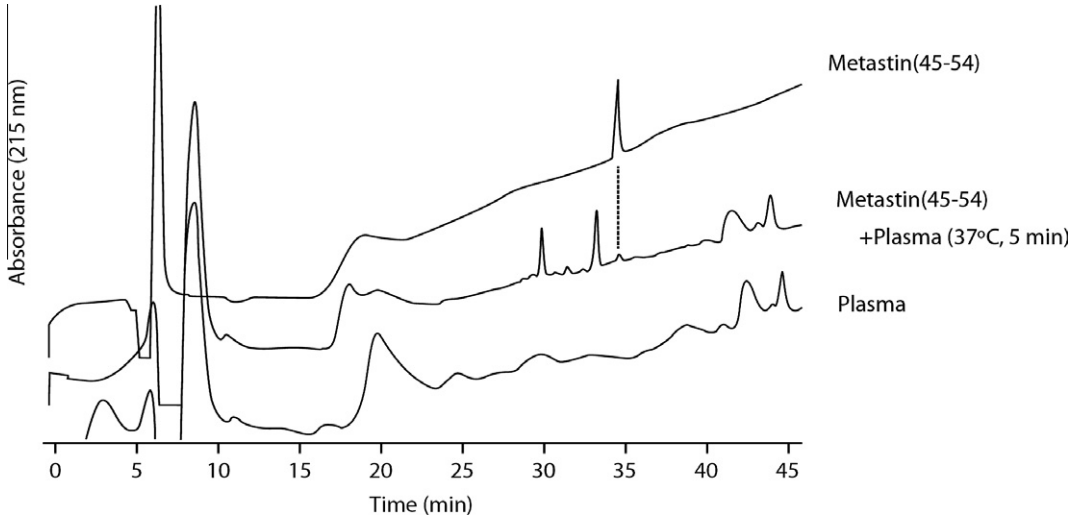

Figure 1. HPLC chromatogram of metastin(45–54) after incubation in mouse plasma.

formed during incubation were identified by HPLC electrospray ionization mass spectrometry (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 as shown in Figure 2. Two major peptide fragments, metastin(46–54) and (46–53), were released primarily as