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SCIENCE DIRECT
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 134–137

Design and synthesis of downsized metastin (45–54) analogs with maintenance of high GPR54 agonistic activity

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Received 22 August 2005; revised 8 September 2005; accepted 12 September 2005
Available online 18 October 2005

Abstract—Metastin has been identified as a metastasis suppressor gene product that mediates its function through a G protein coupled receptor, GPR54. To refine insight into the critical pharmacophore for the activation of GPR54, we have conducted alanine and D-amino acid scanning on a biologically active metastin fragment (45–54). Based on these data and structures of peptides previously reported to activate GPR54, a series of shortened metastin (45–54) derivatives were synthesized and tested for the ability to induce GPR54 signaling. These biological experiments were performed in yeast containing human GPR54 that was coupled to the pheromone response pathway and a pheromone responsive *lacZ* reporter gene. Compounds **32**, **33**, and **39**, which possess an N-terminal basic group and a C-terminal RW-amide motif, were strong agonists, similar to the level of metastin. This may provide an approach to reverse the pro-metastatic effect of metastin deletion in multiple malignant tumors.

A metastasis suppressor gene that was originally designated KiSSl was found to have decreased expression in metastatic melanoma, but not in non-metastatic counterparts.[1] The activity of this gene product was found to be mediated by an orphan GPCR (hOT7T175, AXOR12, GPR54).[2] Metastin, consisting of 54 amino acid residues, was recently identified as an endogenous ligand of GPR54 from a human placental extract.[2a] It was also demonstrated that this peptide was a post-translationally modified derivative of the 145 residues KiSS1/metastin precursor. The biological activity of metastin was localized to a 10 residues (45–54) C-terminally amidated peptide. Metastin (45–54) has been shown to oppose the proliferation and mobility of multiple tumor cell types in vitro. Clinical correlations in human tumors are limited to the finding of an inverse relationship between metastatic potential of malignant melanoma and KiSS1 expression by in situ hybridization.[3]

The biological significance of so-called 'Kisspeptins' is not limited to the negative regulation of the metastatic phenotype. KiSS1/metastin is expressed in placenta at high levels and lower copy numbers are detected in brain and testis.[2] The metastin receptor is expressed in placenta, brain, pituitary, spinal cord, pancreas, and carcinomas of the breast, ovary, and thyroid. Deficiency of metastin receptor function has been associated with hypogonadotropic hypogonadism in humans and genetically modified mouse models.[4]

Thus, metastin may be a candidate for a novel therapy to prevent metastatic spread of multiple malignancies, as well as having implications for hormonal therapy. The therapeutic use of metastin (45–54) possesses many limitations that include limited bioavailability and problematic stability. Downsizing and reduction of the peptide nature of metastin are critical to the future therapeutic targeting of its receptor in malignant and endocrine disorders. In this study we expressed the metastin receptor in yeast coupled to a pheromone-responsive *lacZ* reporter gene to select downsized metastin analogs possessing an N-terminal basic group and a C-terminal RW-amide motif.

Keywords: Metastin; GPR54 agonist.
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doi:10.1016/j.bmcl.2005.09.054

Ohtaki et al. described that N-terminally truncated metastin analog, metastin (45–54), possessed higher affinity for GPR54 compared to full length metastin, but metastin (46–54) was less potent.[2a] They presumed that C-terminally amidated sequence from Tyr^{45} to Phe^{54} is mostly involved in receptor interaction and that N-terminal portion of metastin is not essential for receptor binding, but it may be involved in another biological process such as stabilization and protection from proteolytic digestion. Therefore we envisioned that modification of metastin (45–54) would provide a novel low molecular GPR54 agonist.

First, we carried out Ala-scanning and D-amino acid scanning of metastin (45–54) in order to identify important residues for GPR54-agonistic activity. All peptides were synthesized by standard Fmoc-based solid phase peptide synthesis (Fmoc-SPPS). The activities of the peptides based on the structure of metastin (45–54) were determined in an assay system in yeast in which GPR54 was linked to the pheromone response signaling cascade via a hybrid G alpha subunit, modified from the approach previously described in our laboratory.[5] The agonistic activities of the synthetic peptides were determined by quantification of β-galactosidase activity encoded by a pheromone-sensitive *FUS1-lacZ* reporter gene. Following incubation with the candidate peptides for three hours at 37 °C, lysates were incubated with fluorescein di-β-D-galactopyranoside (FDG) (Molecular Probes, Eugene, OR), a fluorescent β-galactosidase substrate and analyzed in a FUSION (Packard) at appropriate excitation and emission wavelengths. The magnitude of GPR54 activation by the various mutants was compared to that of the metastin (45–54) parental template (Fig. 1).

In the Ala-scanning experiments, substitution of Phe^{50}, Leu^{52} and Phe^{54} resulted in complete loss of agonistic activities (peptides **6**, **8**, and **10**). The D-amino acid scanning showed the importance of stereochemistry of the C-terminal five residues for activation of GPR54 (peptides **16**–**20**). These data suggested that five amino acid residues, Phe^{50} to Phe^{54} play an especially important role for binding and activation of GPR54.

Recently, we reported that cyclic pentapeptide format can serve as an efficient template as a Pharmacophore displaying unit in the development of antagonists of the CXCR4 chemokine receptor.[6] It was reasoned that cyclic peptides containing Phe^{50} to Phe^{54} in metastin (45–54), which were shown to be important for the receptor activation in experiments described above, might show high agonistic activity because of the fixation of active conformation(s) by the ring structure.

We synthesized cyclic peptides **21**–**26** consisting of Ser^{46} to Phe^{54} without exchanging the order of the amino acids (Fig. 2). Gly^{51} was not deleted due to synthetic utility (racemization free, ease of cyclization). Protected peptides were constructed from Gly by Fmoc-SPPS on the 2-chlorotrityl resin. After cleavage from the resin without deprotection of side chain, the resulting peptides were cyclized by use of DPPA. Final deprotection with 95% TFA aq was followed by HPLC purification to yield the desired cyclic peptides. Agonistic activities of these peptides were evaluated by the pheromone-responsive *lacZ* reporter gene assay as described for the alanine scanning mutants. Disappointingly, these peptides showed no agonistic activities for GPR54 in this assay system.

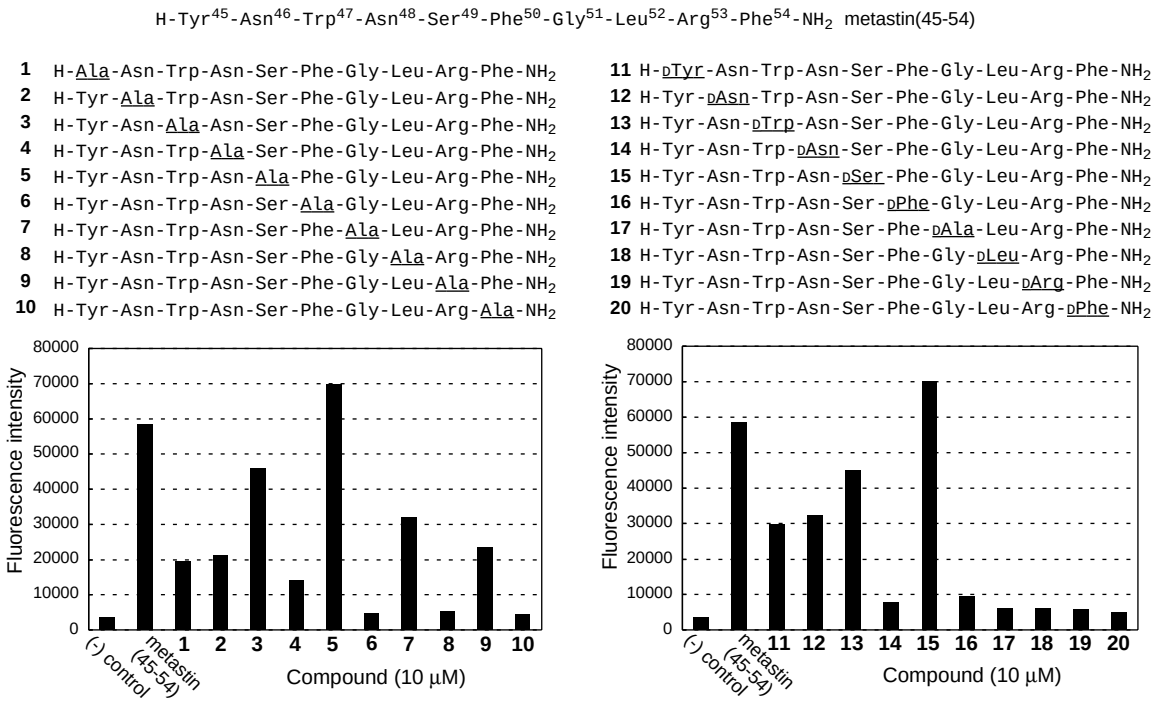


Figure 1. Ala-scanning and D-amino acid scanning of metastin (45–54) by *lacZ* reporter gene assay of GPR54-expressed in yeast.

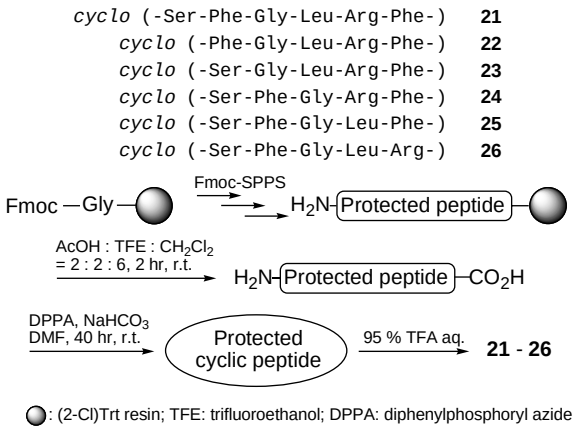


Figure 2. Synthetic scheme of cyclic peptides **21**–**26** and their sequences.

At the same time that metastin was identified as an endogenous ligand for GPR54, it was revealed that several invertebrate neuropeptides also possessed agonistic activity for this receptor. Some of sequences of reported peptides and their EC_{50} values determined by Clements et al. are shown in Table 1.[2b] It was confirmed that the potency of metastin and metastin (45–54) were more active than peptide **27** (reported EC_{50} (μM) value by Muir et al.[2c]: peptide 27: 1.86; metastin: 0.0012; metastin (45–54): 0.00050). The sequences of these peptides are similar to that of the metastin C-terminal region. Comparing peptide **28** with **29**, increased activity was associated with replacement of the C-terminal Phe with Trp. Peptide **30**, which contains an N-terminal side Arg as basic group and G-L-R-W-NH_2 sequence identical to the metastin C-terminus except for Trp residue, was the most potent of these short peptides. Therefore, modifications of metastin (45–54) were performed utilizing these data in order to develop downsized metastin analogs.

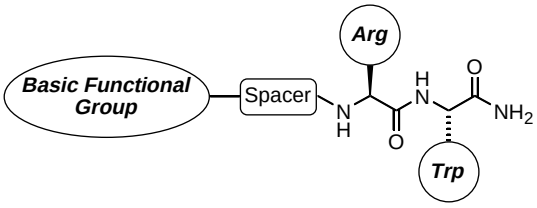
We designed several short metastin derivatives based on the sequence of metastin (45–54) (Fig. 3). All peptides possess an N-terminal basic group, for example a guanide group or a pyridine ring which are often found in GPCR ligands, and the C-terminal RW-amide motif. Several peptides were incorporated DPhe-Pro, Phe-DAla or Phe-DPro sequence as a turn inducing spacer instead of Phe-Gly. All peptides were synthesized by standard Fmoc-SPPS. BisPy group and Gu group were constructed on the resin by reductive amination using 2-pyridinecarboxy aldehyde and $NaBH_3CN$ or commercially available guanilation reagent, respectively (Fig. 4).

The agonistic activities of these peptides were evaluated using the GPR54 pheromone responsive *lacZ* reporter gene in yeast (Fig. 5). In this experiment, peptides **32** (FM052a), **33** (FM053a), and **39** (FM059a) showed high agonistic activities at the same level as full length metastin. The molecular weights of these peptides (**32**: 992; **33**: 852; **39**: 1175) are lower than those of metastin and metastin (45–54) (5857 and 1302, respectively). Thus, the molecular size of metastin was dramatically downsized with maintenance of its agonistic potency. On the other hand, peptides **36**–**38** and **40**–**42**, which contained a turn inducing spacer, were less effective. The efficiencies of turn inducer for improvement of agonistic activity were not observed in this experiment.

In this study, Ala-scanning and D-amino acid scanning of metastin (45–54) were performed to determine the essential structures required for GPR54 agonistic activity. This analysis revealed that the C-terminal five residues of metastin (45–54) were critical to its GPR54 agonistic activity. Next, several metastin derivatives were synthesized based on the structures of metastin (45–54) and invertebrate neuropeptides previously reported as GPR54 agonists. It was demonstrated that significantly downsized analogs, including peptides **32**

Table 1. EC_{50} values of several invertebrate neuropeptides for human GPR54 reported by Clements et al.

Peptide	Sequence	EC_{50} (μM)
27	pEGLRW-NH_2	1.5
28	NRNFLRF-NH_2	8.0
29	NRNFLRW-NH_2	2.1
30	NRNGLRW-NH_2	0.2



31	BisPy-desNLys-Phe-Gly-Leu-Arg-Trp-NH_2
32	BisPy-Amb-Phe-Gly-Leu-Arg-Trp-NH_2
33	Gu-Amb-Phe-Gly-Leu-Arg-Trp-NH_2
34	desNArg-Phe-Gly-Leu-Arg-Trp-NH_2
35	Ac-Arg-Phe-Gly-Leu-Arg-Trp-NH_2
36	Ac-Arg-DPhe-Pro-Leu-Arg-Trp-NH_2
37	Ac-Arg-Phe-DAla-Leu-Arg-Trp-NH_2
38	Ac-Arg-Phe-DPro-Leu-Arg-Trp-NH_2
39	Ac-Trp-Asn-Arg-Phe-Gly-Leu-Arg-Trp-NH_2
40	Ac-Trp-Asn-Arg-DPhe-Pro-Leu-Arg-Trp-NH_2
41	Ac-Trp-Asn-Arg-Phe-DAla-Leu-Arg-Trp-NH_2
42	Ac-Trp-Asn-Arg-Phe-DPro-Leu-Arg-Trp-NH_2

Figure 3. Sequences of short metastin derivatives. Abbreviations: BisPy: bis[(2-pyridinyl)methyl]; desNLys: 6-aminohexanoic acid; Amb: 4-aminomethylbenzoic acid; desNArg: 5-guanidinopentanoic acid; Gu: guanidino.

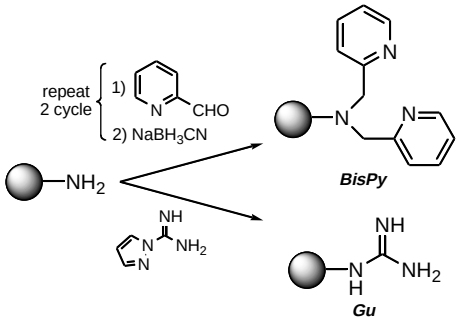


Figure 4. Constructions of BisPy group and Gu group on the resin.

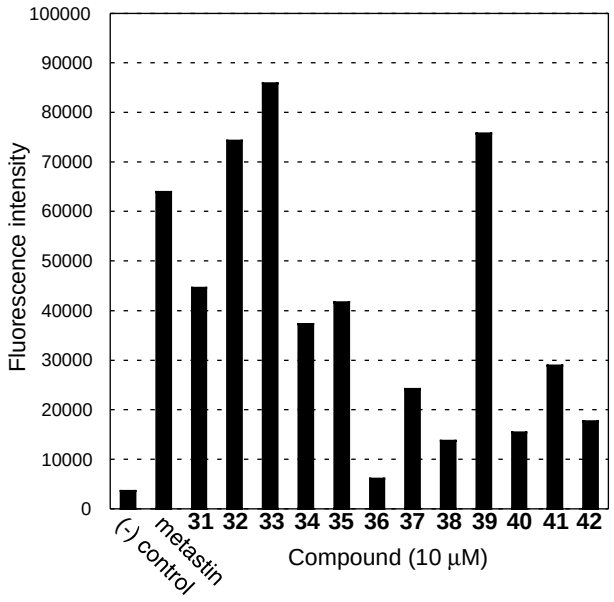


Figure 5. GPR54-agonistic activities of metastin and metastin shortened derivatives **31**–**42** in *lacZ* reporter gene assay on yeast.

(FM052a), **33** (FM053a) and **39** (FM059a), showed high agonistic activity, similar to the potency of metastin. These downsized peptides are potential lead compounds for development of a novel drug targeted to GPR54.

Acknowledgments

This research was supported in part by 21st Century COE Program 'Knowledge Information Infrastructure for Genome Science,' a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan Society for the Promotion of Science (JSPS), and the Japan Health Science Foundation. A.N. is grateful for Research Fellowships of the JSPS for Young Scientists.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.09.054.

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