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New cyclic somatostatin analogues containing a pyrazinone ring: Importance of Tyr for antiproliferative activity

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ABSTRACT

Novel somatostatin analogues containing a pyrazinone ring, compounds **1** and **2**, exhibited good antiproliferative activity on A431 tumor cells. To increase antitumor activity and binding affinity on somatostatin receptors (SSTRs), we substituted Tyr in the critical sequence, Tyr-D-Trp-Lys, with more hydrophobic aromatic residue. The substituted compounds dramatically lost antitumor activity, indicating that Tyr residue was an essential residue.

The neuropeptide somatostatin (SRIF), a tetradecapeptide originally isolated from bovine hypothalamus and identified as a potent inhibitor of GH secretion, exerts a wide variety of inhibitory effects on the endocrine, exocrine, and neural function.[1,2] It also activates tyrosine phosphatases, thus indirectly inhibiting tyrosine kinases which are involved in the regulation of cell proliferation,[3] through a family of seven transmembrane G protein-coupled receptors (SSTR1–SSTR5).[4,5] The distribution of SSTRs ranges widely throughout not only the organs in human body, but also several tumor cells. The peptide-based analogues of somatostatin, such as Octreotide[6] and RC-160,[7] which have high affinity to SSTR2 and SSTR5, have been developed for use in clinical therapies to overcome the short therapeutic half-life of somatostatin, and used in the diagnosis and treatment of gastrointestinal disorders, including endocrine tumors.[6,8] However, the use of these analogues as antitumor agents has been limited because of their antisecretory effects and poor oral bioavailability. Numerous attempts to discover the subtype-selective and orally stable analogues have proceeded; e.g., TT-232, [D-Phe-*c*(Cys-Tyr-D-Trp-Lys-Cys)-Thr-NH_2], was reported to have potent antiproliferative activity without antisecretory action.[9,10] The antiproliferative effect of TT-232 is partially mediated by SSTR receptors (SSTR1 and 4).[11,12] It activates a phosphorylation cascade (the MAP kinase pathway) and results in the induction of cyclin-dependent kinase inhibitors, causing cell cycle arrest. Furthermore, TT-232 might be internalized into the cytoplasm and nuclei, where it directly induces apoptosis.[13] The current study had two objectives. Firstly, to use a pyrazinone ring for greater resistance to enzymatic degradation, thereby enhancing the stability in the body and cellular permeability through the gastrointestinal tract and blood–brain barrier (BBB).[14,15] Secondly, to enhance the antiproliferative activity by increasing the hydrophobicity of the molecule in order to increase interaction between compounds and cell membranes and SSTRs. We previously reported that somatostatin analogues containing pyrazinone rings and active sequence (Tyr-D-Trp-Lys) showed good antiproliferative activities on A431 tumor cells (Fig. 1: **1** and **2**).[16] In that paper, we also used aliphatic amino acids instead of pyrazinone rings to compare the activities between the two. While most cyclic somatostatin analogues have disulfide bonds, these analogues have pyrazinone rings or aliphatic amino acids for their ring formation. As the result, the analogues containing a pyrazinone ring exhibited more potent antiproliferative activity, although both conformations by CD spectra showed random structures.[16] Generally, almost somatostatin analogues form a β-turn structure spanning residues D-Trp and Lys.[17] The pyrazinone ring might allow the proper orientation of active sequence necessary for activity. In fact, we reported the low-energy conformational searching paradigm of a 2′,6′-dimethyl-L-tyrosine (Dmt) dimer ligand containing a pyrazinone ring.[14] The pyrazinone ring as a linker attempts to spatially align two Dmt pharmacophores in order to closely face each other compared with the alkyl linker; therefore, Dmt was well adapted to interact with the opioid receptor.[14] Furthermore, the advantage of a pyrazinone ring is that it is

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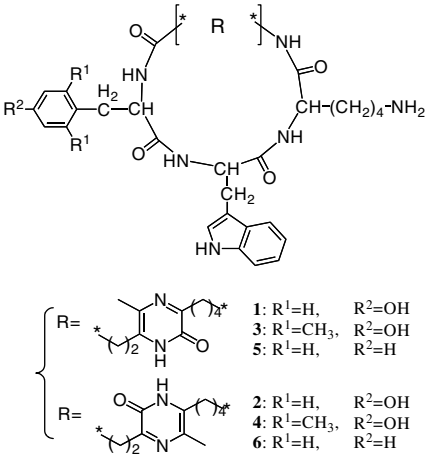


Figure 1. Structures of somatostatin analogues **1–6**. Compounds **1**, **3**, and **5** are [Lys-Glu]-pyrazinone cyclopeptides, compounds **2**, **4**, and **6** are [Glu-Lys]-pyrazinone cyclopeptides.

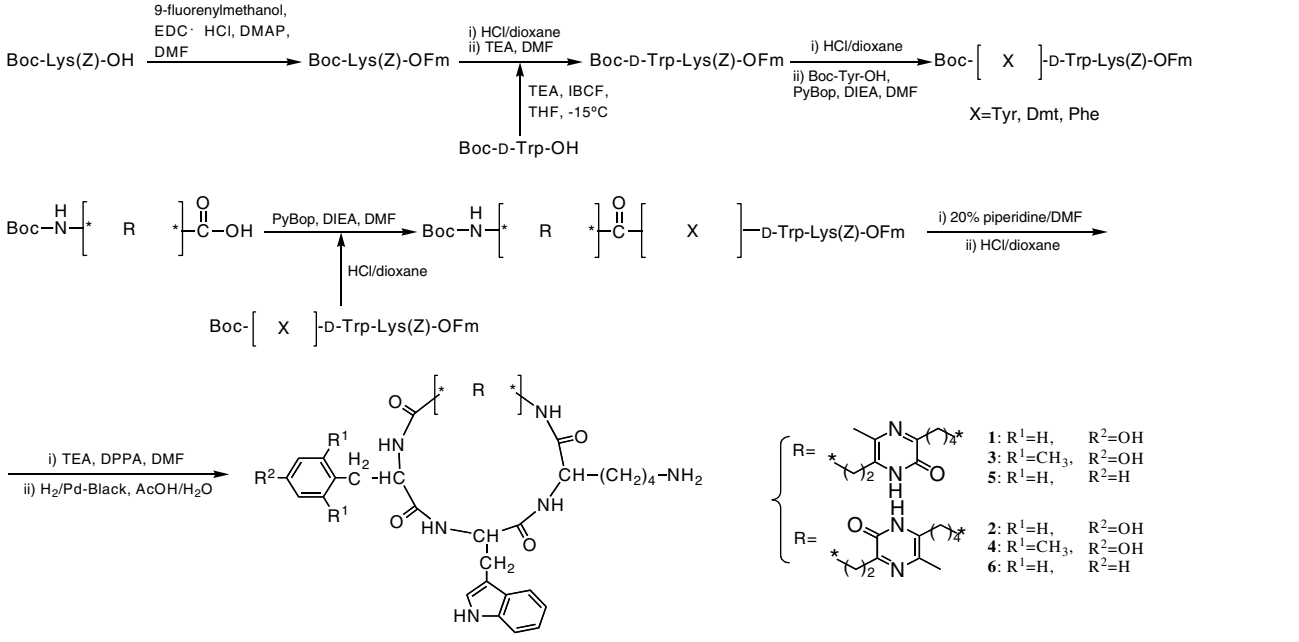
stable in body owing to cyclic structure and allows these compounds to permeate the epithelial cell membranes in the gastrointestinal tracts and BBB.[14,18,19] Thus, somatostatin analogues containing pyrazinone ring should increase the bioavailability in human body. As another approach, the cyclic analogues (**3–6**) were prepared by substituting Tyr with Dmt or Phe in order to improve the biological activity of **1** and **2**. It is well known that Dmt is more hydrophobic than Tyr and its replacement for Tyr in opioid peptides and opioidomimetics dramatically enhanced opioid properties and biological activities in vitro and in vivo.[15,20–22] We postulated that these molecules should exhibit potent antiproliferative activity and bioavailability.

The unnatural amino acid, Dmt was synthesized in our laboratory according to the procedure of Dygos et al.[23] Enantiomeric purity (>98%) was ascertained both by HPLC using a chiral column [CROWNPAC CR(+)] and by enzymatic reaction with L- and D-amino acid oxidases, followed by amino acid analysis. Two pyrazinone derivatives, 3-(4′-aminobutyl)-6-(2′-carboxyethyl)-5-methyl-2(1*H*)-pyrazinone (abbreviated as [Lys-Glu]) and 6-(4′-aminobutyl)-3-(2′-carboxyethyl)-5-methyl-2(1*H*)-pyrazinone, (abbreviated as [Glu-Lys]) were synthesized according to published procedures.[14] Compounds **3–6** were similarly prepared using the procedure reported previously[16] with one modification; namely, Dmt or Phe was used instead of Tyr. Briefly, as shown in Scheme 1, the respective pyrazinone derivatives, Boc-[Lys-Glu]-OH or Boc-[Glu-Lys]-OH intermediate was coupled with H-Dmt/Phe-D-Trp-Lys(Z)-OFm by use of benzotriazol-1-yloxy-tripyrrolidinophosphonium hexafluorophosphate (PyBop) in *N,N*-dimethylformamide (DMF) containing *N,N*-diisopropylethylamine (DIEA). After the removal of 9-fluorenyl methyl ester (OFm) group by 20% piperidine/DMF and *tert*-butyloxycarbonyl (Boc) group by HCl in dioxane, the resulting compounds with free amino and carboxyl groups were purified by semi-preparative reversed-phase (RP)-HPLC. The purified compounds were cyclized using diphenylphosphoryl azide (DPPA) in 1 mM DMF containing triethylamine (TEA). After removal of the benzyloxycarbonyl (Z) group by catalytic hydrogenation in 50% acetic acid (AcOH) for 1 h, the crude products were purified by semi-preparative RP-HPLC. The final compounds were analyzed by MALDI-TOF mass spectrometry, ^{1}H and ^{13}C NMR, elemental analysis.[24]

Table 1
Antiproliferative activity on A431 and SW480 cells by MTT assay.

Compound	A431[a]			SW480[a]		
	10 μM	25 μM	50 μM	10 μM	25 μM	50 μM
TT-232	50 ± 9.4	60 ± 8.5	80 ± 7.4	13 ± 6.5	47 ± 0.7	88 ± 2.6
Cyclohexiinide	40 ± 4.6	70 ± 4.9	80 ± 3.1	68 ± 1.0	85 ± 2.6	92 ± 1.0
1	19 ± 3.0	36 ± 5.0	56 ± 35	24 ± 5.7	23 ± 1.5	7 ± 3.7
2	28 ± 9.0	42 ± 3.0	58 ± 36	35 ± 4.6	31 ± 8.9	35 ± 4.7
3	0 ± 1.9	0 ± 1.2	0 ± 4.5	8 ± 3.7	16 ± 1.0	14 ± 2.0
4	0 ± 4.3	0 ± 2.5	0 ± 2.5	2 ± 5.0	11 ± 3.2	7 ± 7.9
5	31 ± 2.3	28 ± 6.7	25 ± 6.4	0 ± 5.5	7 ± 3.3	0 ± 5.6
6	0 ± 6.8	0 ± 4.9	0 ± 2.3	2 ± 3.8	4 ± 4.5	4 ± 3.6

[a] Antiproliferative activity of compounds are expressed as inhibition of cellular proliferation (%) and listed as the mean ± SE.



Scheme 1. The synthetic route to cyclic compounds **1–6**.

Their antiproliferative activities on A431 cells (human epithelial tumor cells) and SW480 cells (human colon carcinoma cells) on which the SSTRs are expressed were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Table 1).[25] Each analogue was tested at concentrations of 10, 25, and 50 μM. TT-232 and cycloheximide, used as external standards as a well known and efficient apoptosis inducer and cell proliferation inhibitor were used as positive controls.

In comparison with the compounds containing Tyr (**1** and **2**), the replacement by Dmt (**3** and **4**) or Phe (**5** and **6**) resulted in a severe or total loss of antiproliferative activity on A431 cells. Although Dmt-containing opioid peptides and opioidomimetics showed enhanced opioid receptor affinity and biological activity, the decreased antiproliferative activity in the current study indicates that the structures of the binding site of these receptors are quite different from those of opioid receptors in spite of comprising the same G protein-coupled receptor family. The hydrophobicity of compounds were measured by RP-HPLC and the result was Phe (**5** and **6**) > Dmt (**3** and **4**) > Tyr (**1** and **2**) (data not shown). The more hydrophobic compounds had almost no activity, though the ligand–receptor interaction might be activated by hydrophobicity. On the other hand, it may also be conjectured that Tyr interacts with SSTRs through H bonding through the OH functionality. However, the binding site of SSTRs was not accommodated by the added bulkiness of Dmt, unlike opioid receptors. On SW480 cells the activities of all compounds were not much different, but the compounds containing Tyr exhibited more potent activity as expected. The difference of potency between both cells might be due to the number of SSTRs expressed on respective tumor cells and the SSTRs subtype. These results suggest that the Tyr residue in structure of the cyclic peptides **1** and **2** is crucial for antitumor activity.

In conclusion, the newly designed and synthesized somatostatin analogues contained the cyclic conformation due to a pyrazinone ring and the biologically essential amino acid sequence in TT-232. Their antiproliferative activities were tested on two different tumor cell lines, which initially indicated that Tyr, rather than the more hydrophobic residues Dmt or Phe, was an important element. We conclude that the exhibition of antiproliferative activity through SSTRs needs not only hydrophobicity, but also a Tyr residue. The three amino acid residues, Tyr-D-Trp-Lys is surely useful for the design of somatostatin analogues with antitumor activity, and we intend to develop this sequence as core template.

Acknowledgments

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24. Physicochemical data of compounds **3–6**. *c*{[Lys-Glu]-Dmt-D-Trp-Lys}·HCl (**3**): Yield 41.6 mg (78.0%), amorphous, $[\alpha]_D^{25}$ +121° (*c* 1.0, H_2O), R_f 0.71 (*n*-butanol/AcOH/pyridine/H_2O = 4:1:1:2), TOF-MS *m/z*: Calcd $[M+H]^+$. 741.9. Found: 741.9. Anal. Calcd for $C_{40}H_{53}ClN_8O_6{\cdot}4H_2O$: C, 56.6; H, 7.24; N, 13.2. Found. C, 56.6; H, 6.86; N, 13.5. ^{1}H NMR (500 MHz, dimethyl sulfoxide-d_6 (DMSO-d_6)) δ 10.7 (1H, s), 9.08 (1H, s), 8.23 (1H, d, *J* = 8.3 Hz), 7.97 (1H, d, *J* = 8.1 Hz), 7.88 (1H, d, *J* = 8.6 Hz), 7.73 (2H, br), 7.69 (1H, t, *J* = 7.1 Hz), 7.54 (1H, d, *J* = 7.9 Hz), 7.32 (1H, d, *J* = 8.1 Hz), 7.06 (1H, t, *J* = 8.0 Hz), 7.00–6.98 (2H, m), 6.36 (2H, s), 4.64 (1H, q-like, *J* = 7.9 Hz), 4.53 (1H, q-like, *J* = 7.7 Hz), 4.00 (1H, td, *J* = 14 and 8.4 Hz), 3.17 (1H, dq, *J* = 15 and 7.2 Hz), 2.85–2.78 (3H, m), 2.75–2.58 (6H, m), 2.58–2.37 (3H, m), 2.25–2.20 (1H, m), 2.18 (6H, s), 2.12 (3H, s), 1.55–1.45 (2H, m), 1.45–1.27 (4H, m), 1.25–1.15 (2H, m), 0.95–0.85 (2H, br).
c{[Glu-Lys]-Dmt-D-Trp-Lys}·HCl (**4**): Yield 26.5 mg (61.5%), amorphous, $[\alpha]_D^{25}$ +85.8° (*c* 1.0, H_2O), R_f 0.61 (*n*-butanol/AcOH/pyridine/H_2O = 4:1:1:2), TOF-MS *m/z*: Calcd $[M+H]^+$. 741.9. Found: 741.7. Anal. Calcd for $C_{40}H_{53}ClN_8O_6$ $3.5H_2O$: C, 57.2; H, 7.19; N, 13.3. Found: C, 57.0; H, 6.89; N, 13.3. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.7 (1H, s), 8.23 (1H, d, *J* = 7.5 Hz), 8.01 (1H, d, *J* = 8.6 Hz), 7.85 (1H, d, *J* = 6.9 Hz), 7.74 (2H, br), 7.67 (1H, br), 7.55 (1H, d, *J* = 7.9 Hz), 7.31 (1H, d, *J* = 8.1 Hz), 7.05 (1H, t, *J* = 7.6 Hz), 6.97 (1H, t, *J* = 7.5 Hz), 6.94 (1H, d, *J* = 2.0 Hz), 6.36 (2H, s), 4.78 (1H, br), 4.70 (1H, q-like, *J* = 7.7 Hz), 4.02 (1H, q-like, *J* = 7.0 Hz), 3.64–3.55 (1H, m), 2.96–2.86 (1H, m), 2.85–2.72 (3H, m), 2.70–2.61 (3H, m), 2.60–2.48 (3H, m), 2.54 (6H, s), 2.24–2.12 (2H, m), 1.89 (3H, s), 1.56–1.47 (1H, m), 1.46–1.32 (6H, m), 1.28–1.18 (1H, m), 1.08–0.94 (2H, m).
c{[Lys-Glu]-Phe-D-Trp-Lys}·HCl (**5**): Yield 43.5 mg (61.7%), amorphous, $[\alpha]_D^{25}$ +77.8° (*c* 1.0, H_2O), R_f 0.65 (*n*-butanol/AcOH/pyridine/H_2O = 4:1:1:2), TOF-MS *m/z*: Calcd $[M+H]^+$. 697.8. Found: 697.8. Anal. Calcd for $C_{38}H_{49}ClN_8O_5$ $4H_2O$: C, 56.7; H, 7.13; N, 13.9. Found: C, 56.5; H, 7.03; N, 14.1. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.8 (1H, s), 8.48 (1H, d, *J* = 8.1 Hz), 8.24 (1H, d, *J* = 8.2 Hz), 7.83 (1H, d, *J* = 8.3 Hz), 7.75 (2H, br), 7.69 (2H, br), 7.33 (1H, d, *J* = 8.1 Hz), 7.17 (1H, d, *J* = 1.6 Hz), 7.12–7.05 (4H, m), 7.01 (1H, t, *J* = 7.5Hz), 6.93 (2H, d-like, *J* = 6.7 Hz), 4.63–4.54 (2H, m), 4.12 (1H, dt, *J* = 8.5 and 5.7 Hz), 3.17 (1H, m), 3.02 (1H, dd, *J* = 14 and 5.4 Hz), 2.93 (1H, m), 2.86–2.75 (2H, m), 2.74–2.65 (3H, m), 2.57–2.37 (5H, m), 2.24 (1H, s), 2.10 (3H, s), 1.60–1.41 (5H, m), 1.40–1.30 (2H, m), 1.25 (1H, m), 1.07 (2H, m).
c{[Glu-Lys]-Phe-D-Trp-Lys}·HCl (**6**): Yield 45.5 mg (57.3%), amorphous, $[\alpha]_D^{25}$ +41.9° (*c* 1.0, H_2O), R_f 0.48 (*n*-butanol/AcOH/pyridine/H_2O = 4:1:1:2), TOF-MS *m/z*: Calcd $[M+H]^+$. 697.8. Found: 697.5. Anal. Calcd for $C_{38}H_{49}ClN_8O_5{\cdot}3H_2O$: C, 58.0; H, 7.04; N, 14.2. Found: C, 57.9; H, 6.78; N, 14.5. ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.7 (1H, s), 8.23 (1H, d, *J* = 7.5 Hz), 8.01 (1H, d, *J* = 8.6 Hz), 7.85 (1H, d, *J* = 6.9 Hz), 7.74 (2H, br), 7.67 (1H, br), 7.55 (1H, d, *J* = 7.9 Hz), 7.31 (1H, d, *J* = 8.1 Hz), 7.05 (1H, t, *J* = 7.6 Hz), 6.97 (1H, t, *J* = 7.5 Hz), 6.94 (1H, d, *J* = 2.0 Hz), 6.36 (2H, s), 4.78 (1H, br), 4.70 (1H, q-like, *J* = 7.7 Hz), 4.02 (1H, q-like, *J* = 7.0 Hz), 3.64–3.55 (1H, m), 2.96–2.86 (1H, m), 2.85–2.72 (3H, m), 2.70–2.61 (3H, m), 2.60–2.48 (3H, m), 2.54 (6H, s), 2.24–2.12 (2H, m), 1.89 (3H, s), 1.56–1.47 (1H, m), 1.46–1.32 (6H, m), 1.28–1.18 (1H, m), 1.08–0.94 (2H, m).
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