Inhibitory Effect of Somatostatin Peptide Analogues on DNA Polymerase Activity and Human Cancer Cell Proliferation

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Abstract. Background and Objectives: It was previously reported that ten small peptides derived from TT-232, somatostatin structural analogue (compounds 1-10), synthesised by a solution-phase method, exhibited potent antitumour activity on human epithelial tumour (A431) cells. Materials and Methods: The present study investigated the inhibitory activity of these peptide compounds against DNA polymerase (pol) and human cancer cell growth. Results: Among the compounds tested, compounds 1-5, which contain a t-butyloxycarbonyl (Boc) group, inhibited the activity of mammalian pols. Compounds 2 (Boc-Tyr-D-Trp-1-adamantylamide) and 3 (Boc-Tyr-D-Trp-2-adamantylamide) strongly suppressed the growth of a human colon carcinoma (HCT116) cell line and also arrested HCT116 cells in S phase, suggesting that these phenomena observed in cancer cells may be due to the selective inhibition of mammalian pols, especially DNA replicative pol α, by these compounds. Compound 2 induced apoptosis of the cells, although compound 3 did not. Conclusion: Compounds 2 and 3 had an enhanced anticancer effect based on pol inhibition.

Among DNA metabolic enzymes, DNA polymerase (pol) catalyses the addition of deoxyribonucleotides to the 3’-hydroxyl terminus of primed double-stranded DNA (dsDNA) molecules (1). The human genome encodes 15 pols that conduct cellular DNA synthesis (2). Eukaryotic cells reportedly contain the following three replicative types: (i) pols α, δ and ε, (ii) mitochondrial pol γ, and (iii) thirteen repair types, namely pols β, δ, ε, ζ, η, θ, τ, χ, λ, μ and ν, REV1 and terminal deoxynucleotidyl transferase (TdT) (3). DNA metabolic enzymes such as pols are not only essential for DNA replication, repair and recombination, but are also involved in cell division. Selective inhibitors of these enzymes are considered as a group of potentially useful anticancer and anti-parasitic agents, because some suppress human cancer cell proliferation and have cytotoxicity (4-6).

Somatostatin-14 (SRIF), H-Ala-Gly-c(Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys)-OH, a natural tetradecapeptide that inhibits the secretion of a wide variety of growth hormones (GHs), including glucagons, insulin, gastrin and secretin, also affects the regulation of cell proliferation, among others (7-9). These SRIF actions are mediated by somatostatin receptors 1-5 (SSTR1-SSTR5), which are found not only on organs in the human body but also on several tumour cell types (10, 11).

Owing to the wide distribution of SRIF and SSTRs in the central nervous system and the spinal cord, SRIF may play a very important role in neutral transmission. Therefore, the structure–activity relationships of SRIF analogues have been studied in order to clarify SRIF function and to develop clinical applications. For example, octreotide (12), H-D-Phe-c(Cys-Phe-D-Trp-Lys-Thr-Cys)-Thr-ol, is known as an agent for diagnosis and treatment of gastrointestinal disorders, including endocrine tumours (12-16). However, its use as an antitumour agent has been limited because of its anti-secretory effects and poor oral bioavailability. As a result, a key objective in developing new somatostatin analogues with antitumour activity is to find analogues showing selectivity for individual SSTRs and resistance to enzymatic degradation. In a search for potent antitumour somatostatin analogues, Kéri et al. found the somatostatin structural derivative TT-232, H-D-Phe-c(Cys-Tyr-D-Trp-Lys-Cys)-Thr-NH₂, which exhibits
strong anti-tumour activity in vitro and in vivo without other SRIF effects, including anti-secretory action (17, 18). Subsequently, cyclic and linear somatostatin analogues based on the Tyr-D-Trp-Lys active sequence of TT-232 were developed and improved in order to produce more potent, highly selective and stable antitumour agents (19-21). Among these analogues, the small molecule compounds 2-5 and 7-10 were identified (Figure 1), exhibiting potent antitumour activity on A431 tumour cells (human epithelial tumour cells), although compounds 1 and 6, which contain only the important sequence Tyr-D-Trp-Lys, had no activity (19). This was a remarkable finding because most somatostatin analogues that are known to have a highly potent bioactive effect are peptides with a relative large backbone, including the Phe-D-Trp-Lys-Thr sequence cyclised by disulfide bond. In the novel designed compounds 2-5 and compounds 7-10, the C-terminal Lys in the Tyr-D-Trp-Lys active sequence of TT-232 is substituted with hydrophobic and bulky residues (1 or 2-adamantyl, 1-naphthyl or cyclohexyl), and/or there is an induced Boc (t-butyloxy carbonyl) group on the N-terminus, which is considered to improve biological activity and cellular permeability. As expected, a definite correlation between biological activity and hydrophobicity in the whole molecule was demonstrated (19). The dipeptide compounds 2 and 7, and compounds 3 and 8 containing, respectively, 1- or 2-adamantanamine on the C-terminus, exhibited very potent anti-proliferative activity on A431 cells.

The purpose of the present study was to investigate the biochemical action, including inhibition of DNA metabolic enzymes, of the somatostatin peptide analogues compounds 1-10 on in vitro and cellular proliferation processes such as DNA replication of human colon carcinoma (HCT116) cells. Based on the findings of the present study, the potential anti-cancer activity of peptide analogues of somatostatin is also discussed.

Materials and Methods

Materials. The peptide analogues of somatostatin, namely compounds 1-10 (Figure 1), were synthesised by a solution-phase method according to a published procedure (19). The final compounds were purified by semi-preparative reverse-phase HPLC and analysed by MALDI-TOF mass spectrometry, 1H and 13C-NMR and elemental analysis. Chemically synthesised DNA templates, such as poly(dA), and nucleotides, such as [3H]-deoxythymidine 5'-triphosphate (dTTP) (43 Ci/mmol), were obtained from GE Healthcare Bio-Sciences (Little Chalfont, UK). DNA primers, such as oligo(dT)18, were customised by Sigma-Aldrich Japan K.K. (Hokkaido, Japan). All other reagents were of analytical grade and were obtained from Nacalai Tesque, Ltd (Kyoto, Japan). The HCT116 human colon carcinoma cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA).

Enzymes. Pol α was purified from calf thymus by immuno-affinity column chromatography, as described by Tamai et al. (22). Recombinant rat pol β was purified from Escherichia coli JM pβ5, as described by Date et al. (23). The human pol γ catalytic gene was cloned into pFastBac, and the histidine-tagged enzyme was expressed using the BAC-TO-BAC HT Baculovirus Expression System according to the supplier’s manual (Life Technologies, MD, USA) and purified using ProBound resin (Invitrogen Japan, Tokyo, Japan) (24). Human pols δ and ε were purified by the nuclear fractionation of human peripheral blood cancer cells (Molt-4) using affinity column chromatography based on the second subunit of pol

δ and ε, respectively (25). A truncated form of human pol η (residues 1-511) tagged with His6 at its C-terminal was expressed in E. coli cells and purified as described previously (26). A recombinant mouse pol ι tagged with His6 at its C-terminus was expressed and purified by Ni-NTA column chromatography as described elsewhere. A truncated form of pol κ (residues 1-560) with 6× His tags attached at the C-terminus was overproduced in E. coli and purified as described previously (27). Recombinant human His-pol λ was overexpressed and purified according to a method described previously (28). Pol α from a higher plant (cauliflower inflorescence) was purified according to the method outlined by Sakaguchi et al. (29). The Klenow fragment of pol I from E. coli and HIV-1 reverse transcriptase (recombinant) were obtained from Worthington Biochemical Corp. (Freehold, NJ, USA). Taq pol, T4 pol, T7 RNA polymerase and T4 polynucleotide kinase were obtained from Takara (Kyoto, Japan). Calf thymus TdT and bovine pancreas deoxyribonuclease I were obtained from Stratagene Cloning Systems (La Jolla, CA, USA). Purified human placenta topoisomerases (topos) I and II were obtained from TopoGen, Inc. (Columbus, OH, USA). Human telomerase was used for the nuclear fractionation of cultured Molt-4 cells.

Pol assays. The reaction mixtures for calf pol α, rat pol β, plant pol α and prokaryotic mice pol α and β were described previously (30, 31). Those for human pol γ, and human pols δ and ε were as described by Umeda et al. (24) and Ogawa et al. (32), respectively. The reaction mixtures for mammalian pols δ and ε were the same as for calf pol α, and the reaction mixture for human pol λ was the same as for rat pol β. For pols, poly(dA)/oligo(dT)$_{18}$ (A/T=2/1) and dTTP were used as the DNA template-primer and nucleotide [i.e. 2’-deoxyribonucleoside 5’-triphosphate (dNTP)] substrate, respectively. For TdT, oligo(dT)$_{12}$ (3’-OH) and dTTP were used as the DNA primer and nucleotide substrate, respectively.

The compounds were dissolved in high-quality dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 s. Aliquots (4 μl) of sonicated samples were mixed with 16 μl of each enzyme (final amount 0.05 units) in 50 mM Tris-HCl (pH7.5) containing 1 mM dithiothreitol, 50% glycerol and 0.1 mM EDTA, and kept at 0˚C for 10 min. These inhibitor-enzyme mixtures (8 μl) were added to 16 μl of each of the enzyme standard reaction mixtures, and the incubation was carried out at 37˚C for 60 min, except for Taq pol, which was incubated at 74˚C for 60 min. Activity without the inhibitor was considered to be 100%, and the remaining activity at each concentration of the inhibitor was determined relative to this value. One unit of pol activity was defined as the amount of enzyme that catalysed the incorporation of 1 nmol dNTP (i.e. dTTP) into synthetic DNA template primers in 60 min at 37˚C under the normal reaction conditions for each enzyme (30, 31).

Other DNA metabolic enzyme assays. The activities of primase of pol α, human telomerase, HIV-1 reverse transcriptase, T7 RNA polymerase, human topos I and II, T4 polynucleotide kinase and bovine deoxyribonuclease I were measured by standard assays according to the manufacturer’s specifications, as described by Tamiya-Koizumi et al. (33), Oda et al. (34), Ohta et al. (35), Nakayama and Saneyoshi (36), Yonezawa et al. (37), Soltis and Uhlenbeck (38), and Lu and Sakaguchi (39), respectively.

Cell culture and measurement of cell viability. HCT116 cells were cultured in McCoy’s 5A Medium supplemented with 10% foetal bovine serum, penicillin (100 units/ml) and streptomycin (100 mg/ml) at 37˚C in a humid atmosphere of 5% CO$_2$/95% air. For the cell viability assay, cells were plated at 1×10^4 into each well.
of a 96-well microplate with various concentrations of the somatostatin peptide analogues (compounds 1-10). Cell viability was determined by WST-1 assay (40).

Cell cycle analysis. The cellular DNA content for cell cycle analysis was determined as follows: aliquots of 3x10^5 HCT116 cells were harvested into a 35-mm dish, and incubated with a medium containing the somatostatin peptide analogues (compounds 2 and 3) for 24 h. The cells were then washed with ice-cold PBS and collected three times by centrifugation, fixed with 70% (v/v) ethanol, and stored at −20°C. DNA was stained with PI (3, 8-
diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide) staining solution for at least 10 min at room temperature in the dark. Fluorescence intensity was measured by a FACSCanto flow cytometer in combination with FACSDiVa software (BD (Becton, Dickinson and Company), Franklin Lakes, NJ, USA).

Apoptosis assay using immunofluorescence microscopy. Aliquots of 2.5x10^4 cells were plated in each well of an eight-well chamber slide (Nunc, NY, USA). The cells were incubated with compound 2 (15 μM) or compound 3 (35 μM) for 24 h at 37°C. The percentage of apoptotic cells was determined by the ApopTag Red In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA, USA). Apoptotic cells were treated with 25 μM etoposide for 5 h at 37°C. Culture dishes were stained, and the percentage of apoptotic cells was examined under a fluorescence microscope (Olympus IX70; Olympus, Tokyo, Japan).

Results

Effects of somatostatin peptide analogues on mammalian DNA polymerase activity. The chemical structures of the ten peptide analogues of somatostatin (compounds 1-10), which were chemically synthesised, are shown in Figure 1.

Selective inhibitors of mammalian pols are being studied as useful tools and molecular probes to clarify their biological functions, and to develop chemotherapeutic anti-cancer drugs (5, 41). In terms of mammalian pols, pol α, pol β and pol κ have been used as a representative replicative pol (B family pols), a repair/recombination-related pol (X family pols) and a translation synthesis (TLS) repair pol (Y family pols),
respectively (2, 3). Therefore, first, the inhibitory activity of each compound at 100 μM was investigated using calf pol α, rat pol β and human pol κ. As shown in Figure 2, compounds 1-5, which contained the Boc group, inhibited the activity of pols α, β and κ, but the other compounds had little influence on the activity of these pols. Compound 4 showed the strongest inhibition among the ten compounds tested and the somatostatin peptide analogues were ranked in order of their inhibitory effect as follows: compound 4 > compound 3 > compound 2 > compound 1 > compound 5 > compounds 6-10. Among the mammalian pols tested, the inhibitory effect of these compounds was stronger on the activity of pol α than on the activity of pol β or κ. When activated DNA (i.e. DNA digested by bovine deoxyribonuclease I) and dNTP were used as the DNA template primer and nucleotide substrate, respectively, instead of poly(dA)/oligo(dT)_{18} (A/T=2/1) and dTTP, the mode of inhibition by these compounds did not change (data not shown).

Effects of somatostatin peptide analogues on cultured human cancer cell growth. Pols have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents. Peptide analogues of somatostatin may, therefore, be useful in chemotherapy. The cytotoxic effects of the ten compounds were investigated on the human colon carcinoma cultured cell line HCT116. As shown in Figure 3, compound 2 at 50 and 100 μM had the strongest growth inhibitory effect on this cancer cell line among the compounds tested and compound 3 was the second strongest inhibitor. The LD_{50} values of compounds 2 and 3 were 15 and 35 μM, respectively. These compounds have the Boc group and the adamantylamide group, suggesting that both these groups are important for suppressing the growth of human cancer cells. In contrast, compounds 1, 4, 6, 9 and 10 did not prevent human cancer cell growth. In terms of their growth inhibitory effect, the ranking was: compound 2 > compound 3 > compound 5 > compounds 7 and 8 >
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Effects of compounds 2 and 3 on various DNA polymerases and other DNA metabolic enzymes. The inhibition of in vitro DNA metabolic enzyme activity by compounds 2 and 3 was investigated (Table I). Fifteen eukaryotic pols were subclassified into four major families (A, B, X and Y) on the basis of their biochemical properties and amino acid sequence homology (2, 3). Among the 10 mammalian pols tested, compounds 2 and 3 inhibited the activity of DNA replicative pols of the B family, such as pols α, δ and ε, mitochondrial pol γ of the A family, TLS repair-related pols of the Y family (pols η, ι and κ), and DNA repair/recombination-related pol β of the X family. By contrast, these compounds did not influence the activity of pol λ and TdT, which are X family pols. Compound 3 showed 1.1- to 1.5-fold stronger inhibition of mammalian pols than compound 2, and the inhibitory effect of compounds 2 and 3 on the activity of B-family pols (pols α, δ and ε), especially calf pol α, was strongest among the mammalian pols tested. Compounds 2 and 3 had no influence on the activity of plant (cauliflower) pol α or prokaryotic pols (E. coli pol I, Taq pol and T4 pol). Among the other DNA metabolic enzymes tested, these compounds did not inhibit the activity of calf primase of pol α, human telomerase, HIV-1 reverse transcriptase, T7 RNA polymerase, human DNA topoisomerases I and II, T4 polynucleotide kinase or bovine deoxyribonuclease I. As a result, compounds 2 and 3 were shown to be potent and selective inhibitors of mammalian pols, especially DNA replicative pol α.

To determine whether the inhibition resulted from binding to DNA or to the enzyme, the interaction of compounds 2 and 3 with dsDNA was investigated by measuring the thermal transition of dsDNA with or without each compound. The melting temperature (Tm) of dsDNA mixed with an excess amount of these compounds (200 μM each) was measured by using a spectrophotometer equipped with a thermostatic cell holder. In the concentration range used, no thermal transition of Tm was observed; by contrast, 15 μM of ethidium bromide (EtBr), a typical intercalating compound that was used as a
positive control, produced a clear thermal transition. These observations indicated that compounds 2 and 3 did not intercalate to DNA as a template primer; thus, these compounds may bind directly to the enzyme and inhibit its activity.

It was then investigated in more detail whether pol inhibition by compounds 2 and 3 may be effective against human cancer cell proliferation.

**Effects on the cell cycle progression of compounds 2 and 3.** The above results suggested that compounds 2 and 3 inhibit the activity of mammalian pols, and thus it was hypothesised that the selective inhibition of enzymes, such as mammalian pols, may influence cultured cell growth. Therefore, the effect of compounds 2 and 3 on the cell cycle of HCT116 cells was examined by flow cytometry. The cell cycle distribution was recorded after 24 h of treating the cells with each compound at its LD50, and the ratio of cells in the three phases (G1, S and G2/M) in the cell cycle is shown in Figure 4. Compound 2 led to a significant 2.2-fold increase in the proportion of cells in G2/M phase (17.4% to 38.5%) and a 1.6-fold decrease in the population of cells in G1 phase (62.3% to 38.9%) after 24 h (Figure 4B), whereas the proportion of cells in G2/M phase was almost unchanged (20.3% to 22.6%). The effects of compound 3 on the cell cycle showed the same tendency as those of compound 2 (Figure 4C). Aphidicolin, which is an inhibitor of replicative pols (pol α, δ and ε), showed a 1.8-fold increase in cells arrested in S phase (data not shown). These results indicated that the cell cycle arrest of cancer cells caused by these compounds may be caused by the inhibition of pols, especially DNA replicative pol α.

**Effect of compounds 2 and 3 on apoptotic cell death.** To examine whether the S-phase arrest of cells treated with compound 2 or compound 3 was due to apoptosis, DNA strand breaks were analysed by immunofluorescence microscopy. As shown in Figure 5, HCT116 cells treated with compound 3 at its LD50 showed significant DNA strand breaks, and this compound showed an approximately 1.3-fold stronger level of breakage than etoposide, which is a well-known apoptosis inducer. Such foci were barely evident in cells treated with compound 2; therefore, compound 3 may be a stronger inducer of apoptosis than compound 2. The effects of compound 3 must occur in combination with cell cycle arrest and cell death in human cancer cells.

**Discussion**

Because DNA metabolic enzymes are required for DNA synthesis, and cancer cells have a cycle of unregulated DNA replication, many efforts have been made to develop new anti-cancer agents based on DNA metabolic enzyme inhibitors (4, 42). As described in the present study, some peptide analogues of somatostatin, such as compounds 1-5 with Boc group in the X position of Figure 1, inhibited the activity of mammalian pols α, β and x (Figure 2).

Furthermore, compounds 2 and 3 were the first and second strongest cell growth suppressors of the human colon carcinoma cell line HCT116 among the somatostatin peptide analogues (compounds 1-10) tested (Figure 3). These findings suggested that both the Boc group and the adamantylamide group of compounds 2 and 3 may contribute to the suppression of human cancer cell growth. The present results revealed that inhibition of the activity of DNA replicative pols, especially pol α, by these compounds influenced not only cell proliferation but also S-phase arrest during the cell cycle (Figure 4). In HCT116 cells, compound 3 induced apoptosis to a greater level than etoposide, although the apoptotic effect of compound 2 was much less than that of compound 3 (Figure 5), and etoposide. The difference in apoptotic sensitivity between compounds 2 and 3 remains unknown, and their effects on the molecular mechanism of apoptosis will be addressed in further studies. It is possible that treatment with compound 2 may lead to abrupt death; therefore, this compound may strongly inhibit cell growth, causing arrest at S-phase during the cell cycle and scarcely inducing apoptosis.

The three-dimensional structure of compound 2 is shown in Figure 6. The Boc group and the 1-adamantylamide group of this compound are hydrophobic and hydrophilic (high-electronic), respectively. In terms of chemical properties, the present study focused on the calculated log P (CLog P value (partition coefficient for octanol/water) of the ten peptide analogues of somatostatin (Table II). The value of CLog P, which indicates hydrophobicity, for compounds 2 and 3 was in the same range (3.47 and 3.65, respectively); therefore, a CLog P-value of 3.47-3.65 may be essential for inhibition.

Orzeszko et al. reported that adamantane derivatives containing a 1-adamantylamide group or a 2-adamantylamide group have antimicrobial activity (43). The relationship between the inhibitory activity against mammalian pols and the antimicrobial activity of the compounds is unknown at present; future studies may investigate whether there is a link between the antimicrobial mechanism and pol inhibition.

In conclusion, the present study demonstrated that some somatostatin peptide analogues, such as compounds 2 and 3, selectively inhibit the activity of mammalian pols, especially DNA replicative pol α, and suppress human cancer cell proliferation, causing cell cycle arrest and inducing apoptosis in vitro. These compounds therefore should be considered as lead peptides for potentially useful cancer chemotherapy agents. Hence, it is concluded that the peptide analogues of somatostatin are worth investigating further in terms of their in vivo clinical applications in cancer therapy.
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