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Inhibitory effect of novel somatostatin peptide analogues on human cancer cell growth based on the selective inhibition of DNA polymerase β

Isoko Kuriyama [a], Anna Miyazaki [b,c,*], Yuko Tsuda [b,c], Hiromi Yoshida [a], Yoshiyuki Mizushina [a,c,*]

[a] Laboratory of Food & Nutritional Sciences, Faculty of Nutrition, Kobe Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180, Japan
[b] Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Chuo-ku, Kobe, Hyogo 650-8586, Japan
[c] Cooperative Research Center of Life Sciences, Kobe Gakuin University, Chuo-ku, Kobe, Hyogo 650-8586, Japan

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ABSTRACT

The present study was designed to investigate the anticancer activity of novel nine small peptides (compounds **1**–**9**) derived from TT-232, a somatostatin structural analogue, by analyzing the inhibition of mammalian DNA polymerase (pol) and human cancer cell growth. Among the compounds tested, compounds **3** [*tert*-butyloxycarbonyl (Boc)-Tyr-Phe-1-naphthylamide], **4** (Boc-Tyr-Ile-1-naphthylamide), **5** (Boc-Tyr-Leu-1-naphthylamide) and **6** (Boc-Tyr-Val-1-naphthylamide) containing tyrosine (Tyr) but no carboxyl groups, selectively inhibited the activity of rat pol β, which is a DNA repair-related pol. Compounds **3**–**6** strongly inhibited the growth of human colon carcinoma HCT116 $p53^{+/+}$ cells. The influence of compounds **1**–**9** on HCT116 $p53^{-/-}$ cell growth was similar to that observed for HCT116 $p53^{+/+}$ cells. These results suggest that the cancer cell growth suppression induced by these compounds might be related to their inhibition of pol. Compound **4** was the strongest inhibitor of pol β and cancer cell growth among the nine compounds tested. This compound specifically inhibited rat pol β activity, but had no effect on the other 10 mammalian pols investigated. Compound **4** combined with methyl methane sulfonate (MMS) treatment synergistically suppressed HCT116 $p53^{-/-}$ cell growth compared with MMS alone. This compound also induced apoptosis in HCT116 cells with or without p53. From these results, the influence of compound **4**, a specific pol β inhibitor, on the relationship between DNA repair and cancer cell growth is discussed.

1. Introduction

DNA polymerase (pol, DNA-dependent DNA polymerase, E.C.2.7.7.7) 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, ι, κ, 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 also are involved in cell division. Selective inhibitors of these enzymes are considered to be potentially useful anticancer and antiparasitic agents because they have been shown to suppress human cancer cell proliferation and are cytotoxic.[4–6]

Somatostatin-14 (SRIF) is a natural cyclic tetradecapeptide which inhibits the release of growth hormones, suppresses many other bioactive hormones including glucagons, insulin, gastrin and secretin, and also affects the regulation of cell proliferation.[7–9] These diverse pharmacological actions are dependent on their affinities to somatostatin receptors 1–5 (SSTRs1-5), which are widely distributed not only on human tissues including the brain, gastrointestinal tract, and pancreas but also on several cancer cells.[10,11] Somatostatin analogues are used as diagnostic agents and drugs to treat endocrine tumors,[12–15] but their clinical use as antitumor agents is restricted because of their anti-secretory effects and poor oral bioavailability. To clarify the functions of their respective SSTRs and develop more potent analogues, many efforts have been undertaken to identify SSTRs subtype selective analogues. Kéri et al. identified the somatostatin structural derivative TT-232, H-D-Phe-c(Cys-Tyr-D-Trp-Lys-Cys)-Thr-NH_2, which exhibits strong antitumor activity in vitro and in vivo without other SRIF effects, including anti-secretory action.[16,17] Its antitumor activity was not exhibited through interactions with SSTRs although it exhibited a small amount of binding affinity to SSTRs. Previously, we studied the structure-activity relationships of derivatives of Tyr-D-Trp-Lys, the active sequence in TT-232, and identified some compounds with very potent antiproliferative activity toward several cancer cells.[18] Unlike the cyc-

* Corresponding authors. Tel.: +81 78 974 1551x3232; fax: +81 78 974 5689 (Y.M.).
E-mail addresses: miyazaki@pharm.kobegakuin.ac.jp (A. Miyazaki), mizushin@-nutr.kobegakuin.ac.jp (Y. Mizushina).

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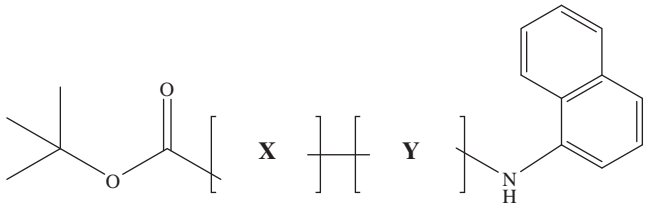
lic compounds reported as general somatostatin analogues, these compounds are small peptide mimetics with features of linear structure and consisting of only two amino acids and one hydrophobic group. Furthermore, the small linear compound *tert*-butyloxycarbonyl (Boc)-Tyr-D-Trp-1-naphthylamide exhibited potent pols (α, β, κ, λ) inhibition, but then showed almost no cancer cell growth inhibitory activity.[19] In this study, we prepared the new small linear compounds **1**–**9** based on the Boc-Tyr-D-Trp-1-naph-



Boc-[X]-[Y]-1-naphthylamide

Compound	X	Y	Compound	X	Y
1	**Tyr**	**Asp**	**5**	**Tyr**	**Leu**
2	**Tyr**	**Glu**	**6**	**Tyr**	**Val**
3	**Tyr**	**Phe**	**7**	—	
			8	—	
4	**Tyr**	**Ile**	**9**	—	

Tyr=

Figure 1. Structures of the synthesized novel peptide analogues of somatostatin. Compound **1**, Boc-Tyr-Asp-1-naphthylamide; compound **2**, Boc-Tyr-Glu-1-naphthylamide; compound **3**, Boc-Tyr-Phe-1-naphthylamide; compound **4**, Boc-Tyr-Ile-1-naphthylamide; compound **5**, Boc-Tyr-Leu-1-naphthylamide; compound **6**, Boc-Tyr-Val-1-naphthylamide; compound **7**, Boc-NH-$(CH_2)_2$-1-naphthylamide; compound **8**, Boc-NH-$(CH_2)_4$-1-naphthylamide and compound **9**, Boc-NH-$(CH_2)_6$-1-naphthylamide.

thylamide sequence and studied their structure-activity relationships. Pols are considered a promising target for anticancer drugs. In particular, pol β plays an important role in base excision repair (BER) of DNA bases damaged by many anticancer drugs and exogenous alkylating agents.[20–22] Pol β is the smallest human pol and the first discovered adaptive polymerase. By X-ray crystallography, it was demonstrated that the full-length pol β protein (39 kDa) was divided into two structural domains, an 8 kDa N-terminal and a 31 kDa C-terminal domain.[23] It is reported that DNA and other pol β inhibitory compounds first bind to the 8 kDa domain binding site,[24,25] which is formed by 4 α-helices, packed as two antiparallel pairs.[26,27] Milon et al. performed docking studies of pol β and pamoic acid, a compound shown to inhibit deoxyribose phosphate lyase and pol β, and found that it bound in a single pocket at the surface of the 8 kDa domain. This pocket contained a positive groove and hydrophobic area where the DNA was also bound.[28] This result promoted us to design novel compounds **1**–**9**. Compounds **1**–**6** contained more hydrophobic and negatively charged amino acids instead of the D-Trp residue found in the parent compound, and compounds **7**–**9** contained only an aliphatic amino acid and a 1-naphthylamide group in order to confirm the necessity of the Tyr-D-Trp sequence.

The biochemical action of the novel somatostatin peptide analogues **1**–**9**, including their in vitro inhibition of mammalian pol species, on DNA replication and repair of human colon carcinoma (HCT116) cells treated with a DNA damaging agent, methyl methane sulfonate (MMS), were studied. The potential anticancer activities of the peptide somatostatin analogues based on their DNA repair-related pol inhibition are discussed. The structures of novel compounds **1**–**9**, all peptide mimetics, make them unique pol inhibitors as other published inhibitors generally contain a hydroxyl group, carbonyl group and aromatic heterocycle.

2. Results

2.1. Effect of somatostatin peptide analogues on mammalian pol activity

The structures of novel nine chemically synthesized somatostatin peptide analogues (compounds **1**–**9**) are shown in Figure 1. The inhibitory activity of each compound at 200 μM was investigated using calf pol α, rat pol β and human pol κ. The mammalian pols α, β and κ were chosen 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] As shown in Figure 2, the activities of pols α and κ were not affected (the pol relative activity was >50%). Compounds **3**–**6**, which contain tyrosine (Tyr) and a hydrophobic amino acid (no carboxyl group), inhibited the activity of pol β. Compound **4** showed the strongest pol β inhibition followed in order by compound **3** > compound **5** > compound **6**.

When activated DNA (bovine deoxyribonuclease I-treated DNA) was used as the DNA template-primer substrate instead of synthesized DNA [poly(dA)/oligo(dT)$_{18}$ (A/T = 2/1)], and dNTP was used as the nucleotide substrate instead of dTTP, the inhibitory effects of these compounds did not change (data not shown).

2.2. Inhibitory effect of compound 4 on eleven mammalian pol species

The activity of compound **4** toward eleven mammalian pols: pol γ (from the A-family), pols α, δ and ε (B-family), pols β, λ, μ and TdT (X-family), and pols η, ι and κ (Y-family) was then investigated.[2,3] Compound **4** dose-dependently inhibited the activity of rat pol β, with an IC$_{50}$ value of 11.5 μM (Table 1). In contrast, compound **4**

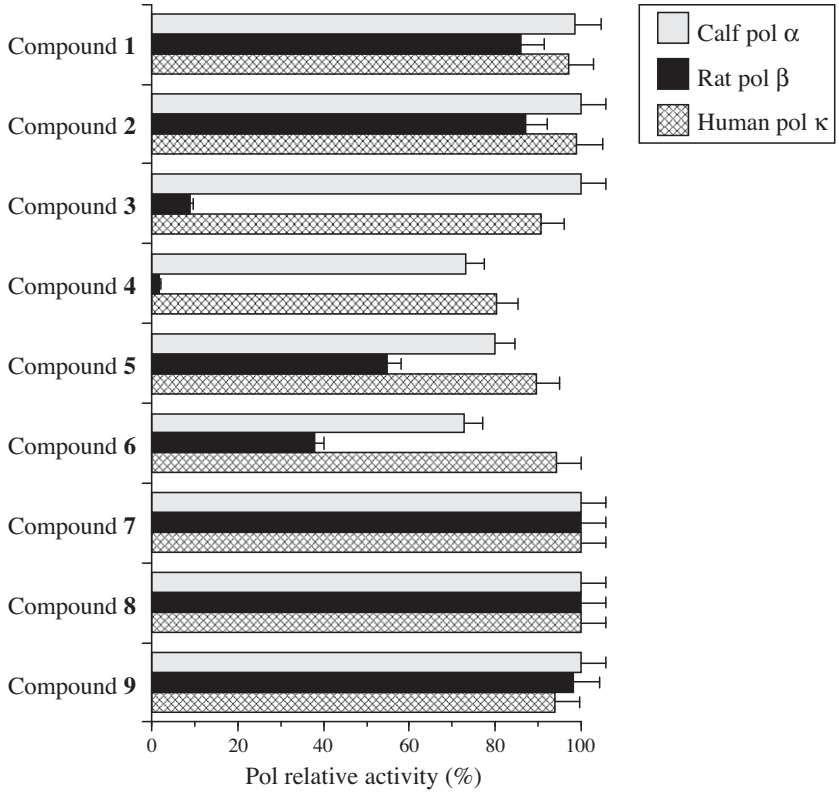


Figure 2. Effect of novel somatostatin peptide analogues (compounds **1**–**9**) on the activity of mammalian pols. Each compound at 200 μM was incubated with calf pol α, rat pol β and human pol κ (0.05 units each). Pol activity was measured as described in the Experimental procedures, and is shown as a percentage of enzyme activity in the absence of the compound, which was taken as 100%. Data are the mean ± S.D. of three independent experiments.

Table 1
Inhibition of mammalian pol activities by compound **4**

Mammalian pols	IC_{50} values (μM)
[A-Family]	
Human pol γ	>200
[B-Family]	
Calf pol α	>200
Human pol δ	>200
Human pol ε	>200
[X-Family]	
Rat pol β	11.5 ± 0.7
Human pol λ	>200
Human pol μ	>200
Calf TdT	>200
[Y-Family]	
Human pol η	>200
Mouse pol ι	>200
Human pol κ	>200

Compound **4** was incubated with each enzyme (0.05 units); pol activity in the absence of inhibitor was set to 100%; data are the mean ±S.D. (n = 3)

exhibited no effect toward the other 10 mammalian pols. In particular, it was interesting that compound **4** did not affect the activities of pol λ, μ and TdT which are thought to share similar homology and three-dimensional structures with pol β, although these pols belong to the X-family.

To ascertain whether the inhibition induced by compound **4** resulted from its ability to bind to DNA or pol β protein, the thermal transition of DNA in the presence or absence of the compound was measured. The interaction of compound **4** with dsDNA was investigated by measuring the melting temperature (Tm) of dsDNA in the presence of excess compound using a spectrophotometer equipped with a thermoelectric cell holder. An alteration in thermal transition (i.e., Tm) was not observed within the compound concentration range tested, whereas a typical intercalating compound used as a positive control (ethidium bromide, 15 μM) produced a clear change in the thermal transition. We also investigated if excess amounts of nucleic acid [poly(rC)] or protein [bovine serum albumin (BSA)] prevented the inhibitory effects of compound **4** in order to determine whether the observed inhibitory effect resulted from non-specific adhesion of these potentially interfering molecules to pol β or from selective binding to specific sites. Poly(rC) and BSA had little or no influence on the inhibitory effects of compound **4** on pol β suggesting that compound **4** bound selectively to the target enzyme molecules. These observations suggested that compound **4** did not act as a DNA intercalating agent but that it bound directly to the enzyme and selectively inhibited its activity.

Taken together, these results suggested that compound **4** was a potent and selective inhibitor of mammalian pol β.

2.3. 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 anticancer agents. The somatostatin peptide analogues (compounds **1**–**9**) could therefore prove useful in chemotherapy. We investigated the cytotoxic effects of the nine compounds against two HCT116 human colon carcinoma cultured cell lines, one wild-type p53 ($p53^{+/+}$) and one deletion mutant of p53 ($p53^{-/-}$). As shown in Figure 3A, 200 μM of compounds **3**–**6** strongly inhibited the growth of HCT116 $p53^{+/+}$ cells. Treatment with compounds **4** and **3** resulted in the strongest cell growth suppression with LD_{50} values of 13.2 and 30.7 μM, respectively. These compounds contain a tyrosine and a hydrophobic amino acid (no carboxyl group), thus the tyrosine group must play a key role in the activities of these compounds. On the other hand, compounds **1**, **2** and **7**–**9** hardly inhibited human cancer cell growth. In terms of the growth inhibitory effect, the ranking was compound **4** > compound **3** > compound **6** > compound **5**. The influence of compounds **1**–**9** on HCT116 $p53^{-/-}$ cell growth showed the same pattern as that observed for HCT116 $p53^{+/+}$ cells (Fig. 3B), suggesting that p53 protein expression had no effect on the cell growth suppression induced by somatostatin peptide analogues. These results suggest that the inhibition of cancer cell growth by these compounds may be related to the inhibition of pol activity, in particular, the inhibition of pol β by compound **4** is likely important for HCT116 cell proliferation. Since compound **4** did not influence the cell growth of a human normal cell line, human dermal fibroblasts (HDF) from the skin (data not shown), this compound might be selective cytotoxic effect against cancer cells. Therefore, additional experiments were carried out on compound **4** in the latter part of this study.

2.4. Reproductive cell death in $p53^{+/+}$ and $p53^{-/-}$ cells

Pol β and p53 play a significant role in base excision repair (BER).[29] Therefore, to determine whether compound **4**-induced cell growth suppression is the cause of the inhibition of pol β, the effects of compound **4** on BER-induced by DNA alkylation damage in HCT116 $p53^{+/+}$ or $p53^{-/-}$ cells were investigated using a clonogenic assay. Alkylation damage induced by MMS is repaired exclusively by the BER pathway.[30] HCT116 cells with or without p53 were treated with 1 mM MMS for 1 h followed by 1% DMSO (vehicle control) or compound **4** at 13.2 μM or 13.7 μM based on the LD_{50} values of $p53^{+/+}$ or $p53^{-/-}$, respectively, for 24 h (Fig. 4). The surviving fraction obtained after 1 mM MMS treatment was 0.05 ± 0.01 and 0.63 ± 0.13 for HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$, respectively, suggesting that HCT116 $p53^{+/+}$ cells could repair the DNA damaged by MMS through a p53 dependent BER pathway, but the HCT116 $p53^{-/-}$ cells could not. When exposed to compound **4**, cell death was slightly favored for HCT116 cells with or without p53. Upon treatment with MMS and compound **4**, p53 wild type cells showed an approximately two fold reduction in clonogenic survival compared with the vehicle control (Fig. 4A). Furthermore, compared to the vehicle control of p53-deficient cells, treatment with compound **4** resulted in a significant reduction (25-fold) in clonogenic survival (Fig. 4B). These data suggested that compound **4** induced cell growth suppression by the inhibition of pol β.

2.5. Effect of compound 4 on apoptotic cell death

To examine whether compound **4** induced apoptosis upon treatment, DNA fragmentation was analyzed by electrophoresis (Fig. 5A). When HCT116 $p53^{+/+}$ cells and HCT116 $p53^{-/-}$ cells were treated with compound **4** at 13.2 μM or 13.7 μM, respectively, for 24 h, these cells clearly underwent DNA fragmentation (lanes 2 and 4 in Fig. 5A). When HCT1116 cells with or without p53 were exposed to 1 mM MMS for 1 h, the observed DNA fragmentation was moderate (lanes 5 and 6 in Fig. 5A). These results suggested that compound **4** and MMS induced apoptosis in both HCT116 $p53^{+/+}$ cells and HCT116 $p53^{-/-}$ cells.

Figure 5B shows the extent of apoptotic cells by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis. The percentage of HCT116 $p53^{+/+}$ apoptotic cells after treatment with compound **4** was three fold higher than that of HCT116 $p53^{-/-}$ cells, suggesting that apoptotic cell death might be induced by a p53 dependent-pathway. MMS also induced apoptosis in HCT116 $p53^{+/+}$ cells, but hardly influenced HCT116 $p53^{-/-}$ cells compared with the control as shown by the high clonogenic sur-

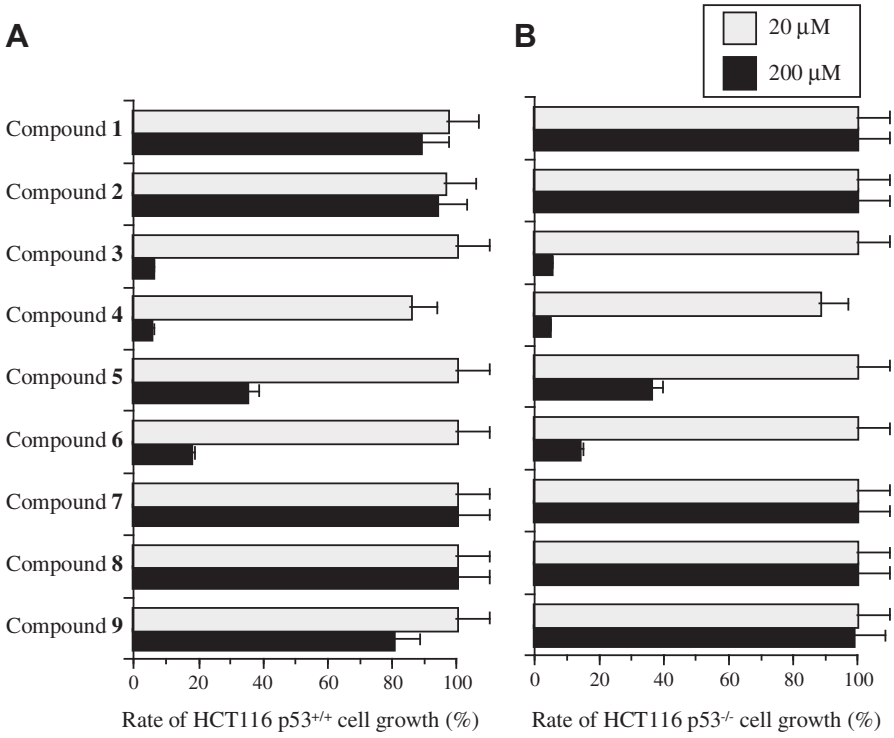


Figure 3. Effect of novel somatostatin peptide analogues (compounds **1**–**9**) on the proliferation of HCT116 human colon carcinoma cultured cell growth. Each compound at 20 μM (gray bar) and 200 μM (black bar) was added to a culture of HCT116 cells with wild-type p53 (HCT116 $p53^{+/+}$) (A) and their isogenic derivatives that lack p53 (HCT116 $p53^{-/-}$) (B). The cells were incubated for 24 h, and the rate of cell growth inhibition was determined by WST-1 assay. Cell growth in the absence of the compound was taken as 100%. Data are the mean ± S.D. of five independent experiments.

vival rate of HCT116 $p53^{-/-}$ cells in the presence of MMS alone (Fig. 4B). Compound **4** was five fold more potent at inducing apoptosis than MMS. Moreover, when MMS and compound **4** were combined together, the extent of apoptotic induction was significantly higher than treatment with either alone for both HCT116 cells with or without p53. This data indicated that compound **4** might further enhance MMS-induced apoptosis.

3. Discussion

We examined the effect of novel nine somatostatin peptide analogues (Fig. 1) on the activities of mammalian pols and human colon carcinoma HCT116 cells with or without p53. Boc-Tyr-Phe-1-naphthylamide (compound **3**) and Boc-Tyr-Ile-1-naphthylamide (compound **4**) potently inhibited the activities of mammalian pols and suppressed human cancer cell growth (Figs. 2 and 3). In particular, compound **4** was a selective inhibitor of pol β (Table 1). Interestingly, these compounds were derived from the parent compound by substituting D-Trp with a more hydrophobic and smaller amino acid. The mechanisms of pol β specific inhibition by compound **4** among the 11 mammalian pols tested remain unclear and will require further study. The suppression of cell growth showed the same trend as the inhibition of pol β among the somatostatin peptide analogues suggesting that the suppression of cell growth is related to the activity of pol β. To determine whether compound **4**-induced cell growth suppression is the result of the inhibition of pol β, the effect of this compound on BER-induced DNA alkylation damage in HCT116 $p53^{+/+}$ or $p53^{-/-}$ cells was investigated using a clonogenic assay. Wild-type p53 protein can significantly stimulate BER in vitro. Furthermore, p53 stabilizes the interaction between pol β and abasic DNA. Hence, after 1 mM MMS treatment, cell survival was 12.6-fold higher for HCT116 $p53^{-/-}$ cells than for HCT116 $p53^{+/+}$ cells (Fig. 4). Cells lacking pol β are highly sensitive to induction of apoptosis and chromosomal breakage by methylating agents such as MMS (Fig. 5). Moreover, incompletely repaired DNA damage causes chromosomal changes and may act as a trigger of DNA damage-induced apoptosis.[31] This phenomenon was observed in the surviving fraction of HCT116 cells without p53 which underwent DNA damage-induced apoptosis to approximately the same extent as HCT116 cells with p53. Recently, interest is growing in inducing apoptosis as a new target for cancer chemotherapy.[32] Apoptosis is an important series of events that leads to programmed cell death and is essential for tissue development and homeostasis. Several studies have indicated that anticancer drugs or cancer chemopreventive agents act by inducing apoptosis in various cancer cells. Additionally, the initiation of apoptosis appears to be a common mechanism of many new chemotherapeutic anticancer agents.[33]

As shown in Figure 5, compound **4** induced apoptosis via a p53-independent pathway in HCT116 cells. The sensitivity of p53 defective cells to the DNA base-damaging agent MMS[34] suggested that certain p53-deficient cancers may be preferentially sensitive to chemotherapeutic base-damaging agents like cyclophosphamide which trigger repair by BER. However, as has been noted by other researchers, there have been surprisingly few studies on the role of p53 in the cellular response to DNA-damaging chemotherapeutic agents for the treatment of epithelial cancers that are not intrinsically prone to undergo apoptosis.[35] Compound **4**, therefore, should be considered a potentially useful anticancer chemotherapeutic agent.

4. Conclusion

The novel somatostatin peptide analogues, especially compound **4**, selectively inhibited the activity of mammalian pol β among the 11 mammalian pol species tested (Fig. 2 and Table 1). The hydrophobic character of the pol β binding site is a very important determinant of its ability to interact with inhibitors and DNA.

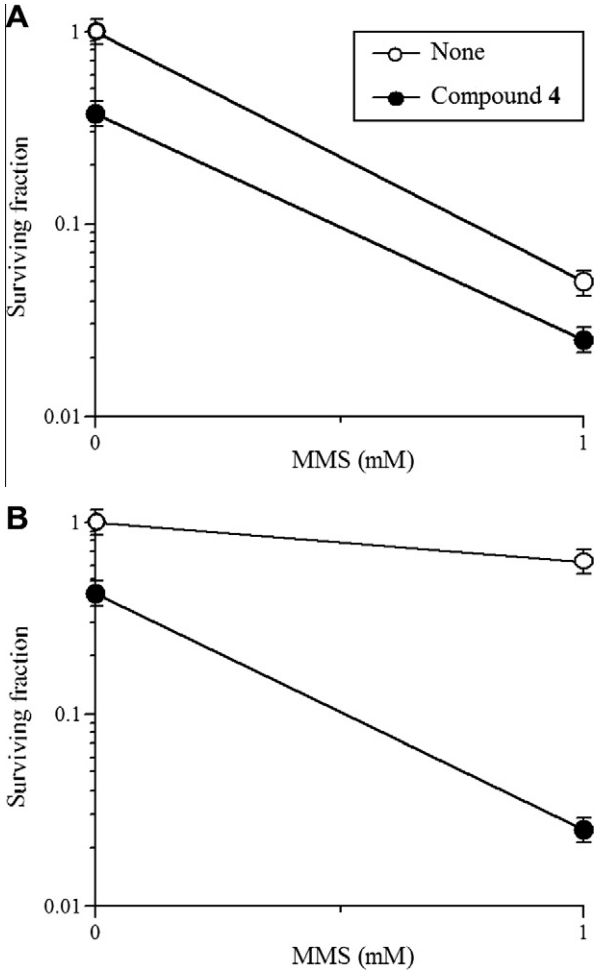


Figure 4. Clonogenic survival of HCT116 cells after treatment with compound **4** and MMS. (A) Treatment schedules combining 1 mM MMS for 1 h and 13.2 μM compound **4** for 24 h of HCT116 $p53^{+/+}$ cells. (B) Treatment schedules combining 1 mM MMS for 1 h and 13.7 μM compound **4** for 24 h of HCT116 $p53^{-/-}$ cells. Cells were treated with (closed circle) or without (open circle) compound **4**. Data are the mean ± S.D. of three independent experiments.

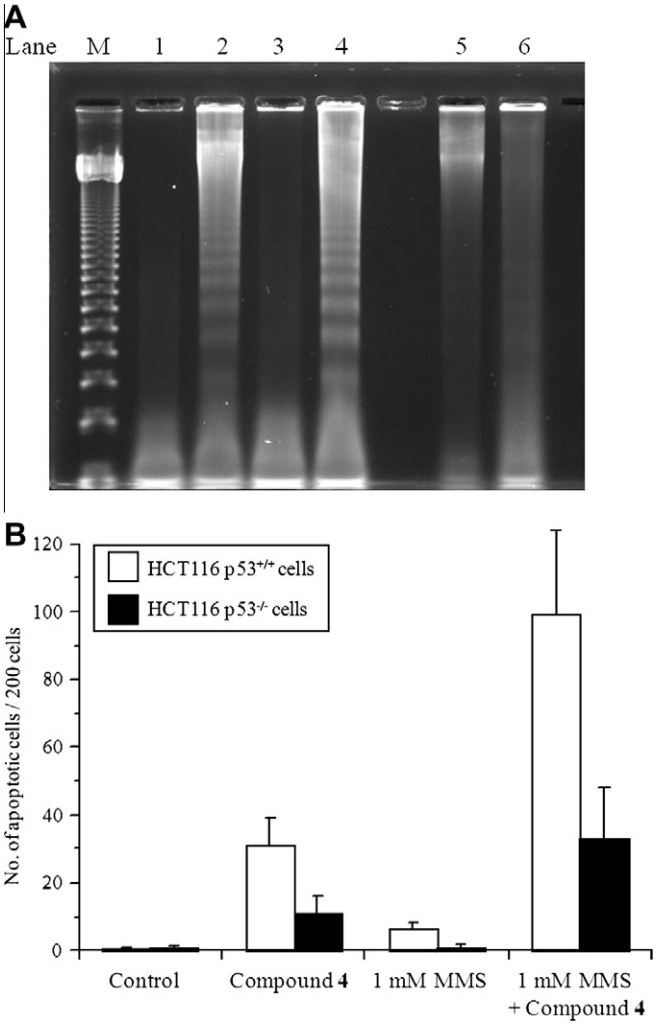


Figure 5. Apoptotic effect of compound **4** or MMS on HCT116 cells. (A) DNA fragmentation of compound **4** or MMS treated cells by agarose gel electrophoresis. M, marker; lane 1, untreated control of HCT116 $p53^{+/+}$ cells; lane 2, HCT116 $p53^{+/+}$ cells were treated with 13.2 μM compound **4** for 24 h; lane 3, untreated control of HCT116 $p53^{-/-}$ cells; lane 4, HCT116 $p53^{-/-}$ cells were treated with 13.7 μM compound **4** for 24 h; lane 5, HCT116 $p53^{+/+}$ cells were treated with 1 mM MMS for 1 h; lane 6, HCT116 $p53^{-/-}$ cells were treated with 1 mM MMS for 1 h. Total DNA was then extracted and analyzed by 1.5% agarose gel electrophoresis. A photograph of the ethidium bromide-stained gel is shown. (B) Apoptotic induction by compound **4** and/or MMS. HCT116 $p53^{+/+}$ cells (gray bar) and HCT116 $p53^{-/-}$ cells (black bar) were incubated for 24 h with 13.2 or 13.7 μM compound **4**, respectively, for 1 h with 1 mM MMS, and for 1 h with 1 mM MMS followed by compound **4** for 24 h (1 mM MMS + compound **4**). Cells were detected by TUNEL assay using an ApopTag Red In Situ Apoptosis Detection Kit. Apoptotic cells were individually counted from a total of at least 200 cells (for each condition). Values are shown as the mean ± S.D. for three independent experiments.

An inhibitor containing a relatively small side chain may be favorable for interaction with the binding pocket. Compounds **1** and **2** contained Asp and Glu, respectively, but showed almost no activity. Other structural features, including the number of carboxyl groups, must play a role in achieving the proper orientation for binding in the pocket, although further study is required to clarify the structure-activity relationship of candidate molecules. Compound **4** potently suppressed human colon carcinoma HCT116 cell growth with or without p53 (Fig. 3). Treatment of HCT116 $p53^{-/-}$ cells with compound **4** significantly enhanced cell multiplication suppression upon pre-addition of MMS (Fig. 4). Therefore, compound **4** has great potential for development as an anticancer agent and could be an effective clinical anticancer chemotherapeutic in combination with DNA damaging agents, such as MMS.

5. Experimental procedure

5.1. General

The chemically synthesized DNA template, poly(dA) was purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). The oligo$(dT)_{18}$ DNA primer was customized by Sigma–Aldrich Japan K.K. (Hokkaido, Japan). The radioactive nucleotide, [^{3}H]-deoxythymidine 5′-triphosphate (dTTP) (43 Ci/mmol), was obtained from MP Biomedicals LLC (Solon, OH, USA). All other reagents were of analytical grade and were purchased from Nacalai Tesque Inc. (Kyoto, Japan). 1-Naphthylamine was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Amino acids and other reagents were purchased from Watanabe Chemical Industries, Ltd (Hiroshima, Japan). Analytical and semi-preparative RP-HPLC columns were Waters 600E with COSMOSIL $5C_{18}$-AR-II (4.6 × 250 mm) and $5C_{18}$-AR-II (20 × 250 mm), respectively. Mobile solvents used were (A) 0.05% TFA in water, (B) 0.05% in acetonitrile, (C) water and (D) acetonitrile. All compounds were analyzed by employing the following gradient program: after maintenance (A):(B) 90:10 for 5 min, and from (A):(B) 90:10 to

10:90 over 40 min. Mass spectra were recorded on a Bruker micro TOF-Q with electrospray ionization (ESI-TOF-MS). ^{1}H NMR spectra were recorded in $CDCl_3$ or DMSO on a Bruker DPX-400 (400 MHz). Silica gel 60 (0.063–0.200 mm, 70–230 mesh by Merck) and Kieselgel G 60 (Merck KGaA, Darmstadt, Germany) were used for open column chromatography and thin layer chromatography, respectively.

5.2. Chemistry

Novel compounds were synthesized by a solution phase method. Starting amino acids and aliphatic amino acids with the *N*-Boc group without a protecting group on the side chain were used, except for Asp and Glu whose side chains were protected by a benzyl ester (OBzl) group. For compounds **1**–**6**, the corresponding amino acid as staring material was coupled with 1-naphthylamine using benzotriazol-1-yloxy-tripyrrolidinophosphonium hexafluorophosphate (PyBOP) in *N*,*N*-dimethylformamide (DMF) containing diisopropylethylamine (DIPEA). For the coupling of Boc-Asp(OBzl)-OH and Boc-Glu(OBzl)-OH, the appropriate amino acid was coupled with 1-naphthylamine by a mixed anhydride method. The Boc group of the resulting compound was removed by 4 M HCl/dioxane to produce the corresponding HCl salt, which was coupled with Boc-Tyr-OH by use of PyBOP to give compounds **3**–**6**. Compounds **1** and **2** were prepared as follows using a further deprotection step. The OBzl group of compounds **1** and **2** was deprotected by catalytic hydrogenation using Pd-Black in 50% AcOH containing a small amount of MeOH for 2 h. For preparation of compounds **7**–**9**, the *N*-Boc forms of 3-aminopropionic acid, 5-aminovaleric acid and 7-aminoheptanoic acid were used as starting materials, and coupled with 1-naphthylamine using a mixed anhydride method. Final products were purified by RP-HPLC and found to be greater than 98% pure. Compounds **1** and **2** were purified using the following gradient program: from (C):(D) 70:30 to (C):(D) 40:60 over 5 min, then (C):(D) 10:90 over 10 min. Compounds **3**–**6** were purified by employing the following gradient program: from (C):(D) 70:30 to (C):(D) 50:50 over 5 min, then (C):(D) 20:80 over 15 min. Compounds **7**–**9** were purified by employing the following gradient program: from (C):(D) 70:30 to (C):(D) 40:60 over 5 min, then (C):(D) 30:70 over 10 min.

5.2.1. General procedure for the synthesis of Boc-[*X*]-[*Y*]-1-naphthylamide [X: Tyr, Y: Asp (1), Glu (2), Phe (3), Ile (4), Leu (5), Val (6)]

Boc-Tyr-OH (720 mg, 2.60 mmol) and H-[*Y*]-1-naphthylamide [prepared from Boc-[*Y*]-1-naphthylamide (1.00 g, 2.60 mmol) and 4 M HCl/dioxane (6.40 mL, 25.6 mmol)] in DMF (50 mL) were coupled by PyBOP (1.47 g, 2.80 mmol) and DIPEA (2.68 mL, 15.3 mmol) at room temperature overnight. After removal of solvent, the residue was extracted with AcOEt, washed with 10% citric acid, 5% $NaHCO_3$ and water, then dried over Na_2SO_4. After removal of Na_2SO_4 by filtration, the filtrate was evaporated. For compounds **1** and **2**, to a solution of the resulting compounds in MeOH (20 mL) containing a drop of H_2O, a small amount of Pd-black was added. H_2 gas was passed through the solution for 2 h. The catalyst was removed by filtration, and the filtrate was evaporated.

5.2.1.1. Boc-Tyr-Asp-1-naphthylamide (1). The residue was precipitated with hexane to give a white solid (40.0%), mp 125–129 °C, R_f 0.46 ($CHCl_3$:MeOH:H_2O = 8:3:1), R_T 34.0 min, ESI-MS *m*/*z* Calcd $[M+H]^+$ 522.22. found 522.22. Anal. Calcd for $C_{28}H_{31}N_3O_7\cdot0.3CH_3CN\cdot2.5MeOH$: C, 60.83; H, 6.88; N, 7.53. Found: C, 60.85; H, 7.05; N, 7.51. ^{1}H NMR ($CDCl_3$) δ 10.21 (s, 1H), 9.19 (br s, 1H), 8.43 (br s, 1H), 8.10 (br s, 1H), 7.93 (t, *J* = 4.7 Hz, 1H), 7.77 (d, *J* = 8.2 Hz, 1H), 7.66 (d, *J* = 7.1 Hz, 1H), 7.54–7.47 (m, 3H), 7.05 (d, *J* = 8.1 Hz, 2H), 6.83 (d, *J* = 7.9 Hz, 1H), 6.62 (d, *J* = 8.1 Hz, 2H), 4.86 (br s, 1H), 4.13 (br s, 1H), 2.91 (br s, 1H), 2.67 (br s, 2H), 1.27 (s, 9H).

5.2.1.2. Boc-Tyr-Glu-1-naphthylamide (2). The residue was precipitated with hexane to give a white solid (45.7%), mp 117–120 °C, R_f 0.51 ($CHCl_3$:MeOH:H_2O = 8:3:1), R_T 33.7 min, ESI-MS *m*/*z* Calcd $[M+H]^+$ 536.23. Found 536.27. Anal. Calcd for $C_{29}H_{33}N_3O_7\cdot0.9H_2O$: C, 63.12; H, 6.36; N, 7.62. Found: C, 63.00; H, 6.26; N, 7.65. ^{1}H NMR ($CDCl_3$) δ 10.05 (s, 1H), 9.19 (br s, 1H), 8.23 (d, *J* = 7.6 Hz, 1H), 8.05 (br s, 1H), 7.94 (td, *J* = 1.9 and 5.9 Hz, 1H), 7.79 (d, *J* = 8.2 Hz, 1H), 7.65 (d, *J* = 7.3 Hz, 1H), 7.57–7.48 (m, 3H), 7.06 (d, *J* = 8.4 Hz, 2H), 6.95 (d, *J* = 8.3 Hz, 1H), 6.63 (d, *J* = 8.4 Hz, 2H), 4.65 (q, *J* = 7.0 Hz, 1H), 4.16 (m, 1H), 2.91 (dd, *J* = 4.0 and 14 Hz, 1H), 2.67 (dd, *J* = 10 and 14 Hz, 1H), 2.42–2.31 (br s, 2H), 2.14 (br s, 1H), 2.10 (br s, 1H), 1.27 (s, 9H).

5.2.1.3. Boc-Tyr-Phe-1-naphthylamide (3). The residue was precipitated with hexane and a small amount of ether to give a white solid (60.0%), mp 207–210 °C, R_f 0.44 ($CHCl_3$:MeOH:AcOH = 90:8:2), R_T 40.3 min, ESI-MS *m*/*z* Calcd $[M+H]^+$ 554.26. Found 554.29. Anal. Calcd for $C_{33}H_{35}N_3O_5\cdot0.1H_2O$: C, 71.35; H, 6.39; N, 7.57. Found: C, 71.30; H, 6.37; N, 7.64. ^{1}H NMR ($CDCl_3$) δ 10.04 (s, 1H), 9.16 (s, 1H), 8.25 (d, *J* = 7.8 Hz, 1H), 7.92 (d, *J* = 7.9 Hz, 1H), 7.76 (t-like, 2H), 7.54–7.45 (m, 4H), 7.37–7.21 (m, 5H), 7.00 (d, *J* = 8.3 Hz, 2H), 6.88 (d, *J* = 8.5 Hz, 1H), 6.62 (d, *J* = 8.3 Hz, 2H), 4.93 (q, *J* = 7.4 Hz, 1H), 4.12 (m, 1H), 3.16 (dd, *J* = 6.7 and 13 Hz, 1H), 3.07 (dd, *J* = 7.9 and 14 Hz, 1H), 2.81 (dd, *J* = 4.1 and 14 Hz, 1H), 2.62 (dd, *J* = 10 and 13 Hz, 1H), 1.29 (s, 9H).

5.2.1.4. Boc-Tyr-Ile-1-naphthylamide (4). The residue was precipitated with ether to give a white solid (89.0%), mp 197–200 °C, R_f 0.48 ($CHCl_3$:MeOH:AcOH = 90:8:2), R_T 39.7 min, ESI-MS *m*/*z* Calcd $[M+H]^+$ 520.27. Found 519.90. Anal. Calcd for $C_{30}H_{37}N_3O_5\cdot1.2H_2O$: C, 66.57; H, 7.34; N, 7.77. Found: C, 66.44; H, 7.29; N, 7.76. ^{1}H NMR ($CDCl_3$) δ 10.12 (s, 1H), 9.16 (s, 1H), 8.06 (br s, 1H), 8.00 (d, *J* = 8.6 Hz, 1H), 7.94 (td, *J* = 1.5 and 5.0 Hz, 1H), 7.79 (d, *J* = 8.2 Hz, 1H), 7.63 (d, *J* = 7.1 Hz, 1H), 7.57–7.49 (m, 3H), 7.38 (d, *J* = 8.4 Hz, 2H), 6.95 (d, *J* = 8.5 Hz, 1H), 6.63 (d, *J* = 8.3 Hz, 2H), 4.60 (t, *J* = 8.2 Hz, 1H), 4.19 (m, 1H), 2.90 (dd, *J* = 4.0 and 7.2 Hz, 1H), 2.69 (dd, *J* = 6.9 and 10 Hz, 1H), 1.92 (br s, 1H), 1.61 (br s, 1H), 1.32–1.27 (m, 10H), 1.02 (d, *J* = 6.7 Hz, 3H), 0.91 (t, *J* = 7.2 Hz, 3H).

5.2.1.5. Boc-Tyr-Leu-1-naphthylamide (5). The residue was precipitated with ether to give a white solid (53.4%), mp 205–209 °C, R_f 0.37 ($CHCl_3$:MeOH:AcOH = 90:8:2), R_T 40.4 min, ESI-MS *m*/*z* Calcd $[M+H]^+$ 520.27. Found 520.31. Anal. Calcd for $C_{30}H_{37}N_3O_5\cdot0.4H_2O$: C, 68.39; H, 7.23; N, 7.98. Found: C, 68.27; H, 7.28; N, 8.11. ^{1}H NMR ($CDCl_3$) δ10.03 (s, 1H), 9.16 (s, 1H), 8.16 (d, *J* = 7.9 Hz, 1H), 8.03 (t-like, 1H), 7.94 (td, *J* = 1.7 and 4.3 Hz, 1H), 7.79 (d, *J* = 8.1 Hz, 1H), 7.61–7.48 (m, 4H), 7.06 (d, *J* = 8.4 Hz, 2H), 6.92 (d, *J* = 8.4 Hz, 1H), 6.62 (d, *J* = 8.4 Hz, 2H), 4.68 (q, *J* = 7.5 Hz, 1H), 4.16 (m, 1H), 2.89 (dd, *J* = 4.0 and 14 Hz, 1H), 2.67 (dd, *J* = 10 and 14 Hz, 1H), 1.78–1.68 (m, 3H), 1.31 (s, 9H), 0.99 (d, *J* = 6.3 Hz, 2H), 0.95 (d, *J* = 6.2 Hz, 2H).

5.2.1.6. Boc-Tyr-Val-1-naphthylamide (6). The residue was precipitated with ether to give a white solid (66.7%), mp 197–200 °C, R_f 0.38 ($CHCl_3$:MeOH:AcOH = 90:8:2), R_T 38.1 min, ESI-MS *m*/*z* Calcd $[M+H]^+$ 506.26. Found 506.26. Anal. Calcd for $C_{29}H_{35}N_3O_5\cdot0.5H_2O$: C, 67.69; H, 7.05; N, 8.17. Found: C, 67.85; H, 7.05; N, 8.29. ^{1}H NMR ($CDCl_3$) δ 10.11 (s, 1H), 9.17 (s, 1H), 8.06 (br s, 1H), 7.97–7.93 (m, 2H), 7.79 (d, *J* = 8.2 Hz, 1H), 7.64 (d, *J* = 7.1 Hz, 1H), 7.58–7.49 (m, 3H), 7.07 (d, *J* = 8.4 Hz, 2H), 6.98 (d, *J* = 8.5 Hz, 1H), 6.64 (d, *J* = 8.4 Hz, 2H), 4.59 (t, *J* = 7.9 Hz, 1H), 4.20 (m, 1H), 2.91 (dd, *J* = 4.0 and 14 Hz, 1H), 2.69 (dd, *J* = 10 and

14 Hz, 1H), 2.21–2.12 (m, 1H), 1.32 (s, 9H), 1.04 (d, J = 6.7 Hz, 3H), 1.00 (d, J = 6.7 Hz, 3H).

5.2.2. General procedure for the synthesis of Boc-NH-$(CH_2)_n$-1-naphthylamide [n = 2 (7), 4 (8), 6 (9)]

To a solution of Boc-NH–$(CH_2)_2$–OH (1.00 g, 4.10 mmol) in tetrahydrofuran (THF) (30 mL), triethylamine (TEA) (0.70 mL, 5.00 mmol) and isobutyl chroloformate (0.60 mL, 4.50 mmol) were added and stirred at −15 °C for 15 min. To this solution, 1-naphthylamine (0.60 g, 4.10 mmol) in DMF (40 mL) containing TEA (0.70 mL, 5.00 mmol) was added and stirred at room temperature overnight. After removal of solvent, the residue was extracted with AcOEt, washed with 10% citric acid, 5% $NaHCO_3$ and water, then dried over Na_2SO_4. After removal of Na_2SO_4 by filtration, the filtrate was evaporated. The residue was precipitated with ether to give a crystalline solid.

5.2.2.1. Boc-NH-$(CH_2)_2$-1-naphthylamide (7). White solid (61.5%), mp 125–130 °C, R_f 0.49 (AcOEt/hexane = 1:1), R_T 35.1 min, ESI-MS m/z Calcd $[M+H]^+$ 315.16. Found 315.19. Anal. Calcd for $C_{18}H_{22}N_2O_3$: C, 68.77; H, 7.05; N, 8.91. Found: C, 68.52; H, 7.24; N, 8.85. ^1H NMR ($CDCl_3$) δ 7.93 (br s, 1H), 7.86–7.82 (m, 3H), 7.70 (d, J = 8.2 Hz, 1H), 7.51–7.41 (m, 3H), 5.23 (br s, 1H), 3.54 (q, J = 5.8 Hz, 2H), 2.73 (t, J = 5.6 Hz, 2H), 1.44 (s, 9H).

5.2.2.2. Boc-NH-$(CH_2)_4$-1-naphthylamide (8). White solid (63.8%), mp 122–127 °C, R_f 0.38 (AcOEt/hexane = 1:1), R_T 37.7 min, ESI-MS m/z calcd $[M+H]^+$ 343.19. Found 343.42. Anal. Calcd for $C_{20}H_{26}N_2O_3$: C, 70.15; H, 7.65; N, 8.18. Found: C, 70.07; H, 7.70; N, 8.19. ^1H NMR ($CDCl_3$) δ 7.96 (br s, 1H), 7.90–7.84 (m, 3H), 7.68 (d, J = 8.1 Hz, 1H), 7.50–7.42 (m, 3H), 4.70 (br s, 1H), 3.19 (br s-q, J = 5.7 Hz, 2H), 2.52 (t, J = 7.2 Hz, 2H), 1.85–1.77 (m, 2H), 1.62–1.55 (m, 2H), 1.44 (s, 9H).

5.2.2.3. Boc-NH-$(CH_2)_6$-1-naphthylamide (9). White solid (82.4%), mp 89–94 °C, R_f 0.60 (AcOEt/hexane = 1:1), R_T 40.4 min, ESI-MS m/z Calcd $[M+H]^+$ 371.23. Found 371.13. Anal. Calcd for $C_{22}H_{30}N_2O_3$: C, 71.32; H, 8.16; N, 7.56. Found: C, 71.56; H, 8.23; N, 7.61. ^1H NMR ($CDCl_3$) δ 7.88–7.84 (m, 3H), 7.80 (br s, 1H), 7.69 (d, J = 8.1 Hz, 1H), 7.50–7.43 (m, 3H), 4.54 (br s, 1H), 3.11 (br s-q, J = 5.4 Hz, 2H), 2.48 (t, J = 7.3 Hz, 2H), 1.80–1.74 (m, 2H), 1.62–1.55 (m, 2H), 1.44 (s, 9H), 1.44–1.35 (m, 6H).

5.3. Pol assays

Mammalian pols with high activities were purified according to our previous report,[36] and standard pol reaction mixtures for pols α and β have been previously described;[37,38] those for pol γ and for pols δ and ε are described by Umeda et al.[39] and Ogawa et al. [40] respectively; for pols η, ι and κ, the method is the same as for pol α; and for pols λ, μ and TdT, the method is the same as for pol β. For pol reactions, poly(dA)/oligo(dT)$_{18}$ (A/T, 2/1) and 2′-deoxythymidine-5′-triphosphate (dTTP) were used as the DNA template-primer substrate and nucleotide (dNTP, 2′-deoxynucleotide-5′-triphosphate) substrate, respectively. For TdT, oligo(dT)$_{18}$ (3′-OH) and dTTP were used as the DNA primer and nucleotide substrate, respectively.

The novel peptide analogues of somatostatin (i.e., compounds **1**–**9**) were dissolved in dimethyl sulfoxide (DMSO) at various concentrations and sonicated for 30 s. Then 4 μL aliquots of the solutions were mixed with 16 μL of each enzyme (final amount 0.05 units) in 50 mM Tris–HCl at pH7.5, containing 1 mM dithiothreitol, 50% glycerol (by vol), and 0.1 mM EDTA, and held 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 incubated at 37 °C for 60 min. Activity without the inhibitor was considered 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 each enzyme that catalyzed the incorporation of 1 nmol of dNTP (i.e., dTTP) into synthetic DNA template-primers in 60 min at 37 °C and under normal reaction conditions.[30,31]

5.4. Cell culture and measurement of cell viability

To investigate the effects of novel somatostatin peptide analogues (compounds **1**–**9**) on cultured human cancer cells, HCT116 human colon carcinoma cells with wild-type p53 (HCT116 p53$^{+/+}$) and their isogenic derivatives lacking p53 (HCT116 p53$^{-/-}$) were used. These two cell lines were a kind gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore). HCT116 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 μg/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 compounds **1**–**9**. Cell viability was determined by the WST-1 assay.[41]

5.5. Colony formation

Reproductive cell death was assayed by measuring colony formation with and without MMS/compound **4**. Cells (from 500 up to 1×10^4 cells per plate, depending on the dose level) were seeded per 10 cm dish and treated with vehicle (DMSO), MMS, compound **4**, or MMS plus compound **4**. Colonies that appeared after 8 days were fixed with methanol, stained with methylene blue, and counted. Relative colony formation (%) was expressed as colonies per treatment level/colonies that appeared in the control.

5.6. Analysis of DNA fragmentation

Apoptosis was determined using a DNA fragmentation assay. Total DNA was extracted from the cultured HCT116 p53$^{+/+}$ and HCT116 p53$^{-/-}$ cells (5×10^5) treated for 24 h with compound **4** (13.2 μM or 13.7 μM, respectively, based on their LD_{50} values) following the method of Sambrook et al.[42] and 5 μg aliquots were separated by 1.5% (w/v) agarose gel electrophoresis in 40 mM Tris-5 mM sodium acetate-1 mM EDTA (pH 7.8) and stained with ethidium bromide. DNA bands were visualized under ultraviolet light.

5.7. Apoptosis assay using immunofluorescence microscopy

Aliquots of 2.5×10^4 cells were plated in each well of an 8-well chamber slide (Nunc, NY, USA). HCT116 p53$^{+/+}$ and HCT116 p53$^{-/-}$ cells were incubated with compound **4** (13.2 μM or 13.7 μM, respectively, based on their LD_{50} values) for 24 h at 37 °C. TUNEL is a method for detecting DNA fragmentation by labeling the terminal end of nucleic acids. The percentage of apoptotic cells was determined using the ApopTag Red In Situ Apoptosis Detection Kit (CHEMICON, 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).

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