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ORIGINAL ARTICLE

Effects of somatostatin analogs octreotide and lanreotide on the proliferation and apoptosis in colon 38 tumor: interaction with 5-fluorouracil

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Abstract

OBJECTIVES: The aim of this study was to compare the effects of two somatostatin (SS) analogs: octreotide and lanreotide administered separately or together with 5-fluorouracil (5-FU) with the action of 5-FU alone, on proliferation, apoptosis, proliferation/apoptosis (P/A) ratio and tumor weight in the murine transplantable Colon 38 cancer.

MATERIALS AND METHODS: Two separate experiments were performed on male mice of B6D2F1 strain. In both experiments the animals were implanted subcutaneously with suspension of Colon 38 cancer cells. A few days later, in the first experiment, octreotide was given once daily for six days at the dose of 10 μg/animal and in the second experiment a single dose of lanreotide (1 mg/animal) was administered. 5-FU was treated once (in the first experiment) or twice (in the second experiment) at the dose of 70 mg/kg b.w. depending on the duration of the experiment. In both experiments the incorporation of bromodeoxyuridine into the tumor cell nuclei was used as an index of cell proliferation (labeling index - LI). The labeling of nuclear DNA fragmentation according to TUNEL method was considered as an index of apoptosis (AI).

RESULTS: It has been found that both SS analogs, given separately, significantly decreased LI of tumor cells, increased their AI and decreased P/A ratio of Colon 38 tumors, as compared to controls. 5-FU given alone (in both experiments) significantly enhanced AI, decreased P/A ratio, but did not significantly change LI, as compared to controls. The joint treatment with 5-FU and each of the two SS analogs did not produce any additive effects either on proliferation or apoptosis. The tumor weight was significantly decreased only by the joint treatment with 5-FU and octreotide, as compared to the control group.

Introduction

It is well-known that somatostatin (SS) analogs can influence the growth processes in normal and neoplastic tissues, mainly via inhibition of cellular proliferation [1, 2, 3]. These inhibitory effects of SS analogs on the proliferation of various lines of colorectal cancer have been shown in both human and animal origin [4, 5, 6]. In our earlier study [6] we found that administration of octreotide in vivo resulted in the inhibition of proliferation and induction of apoptosis of Colon 38 cancer cells. Other authors [7] have observed that octreotide enhanced the inhibitory effect of 5-fluorouracil (5-FU) on ^{3}H-thymidine uptake in two human colon cancer cell lines in vitro, but had no effect on cell growth when administered alone. Similarly, in our previous study [8] we have noticed that octreotide given together with 5-FU caused a more pronounced inhibitory effect on proliferation of Colon 38 cancer and a more pronounced reduction in the tumor mass than in the group treated with 5-FU alone. Chen et al. [9] observed that octreotide modulated the 5-FU incorporation into macromolecules. These findings taken together suggested that octreotide may act on colonic cancer growth synergistically with 5-FU. 5-FU, despite being the most effective current chemotherapeutic treatment in patients with colorectal cancer, has had only poor clinical effectiveness (response rates of only 5-20%). The clinical trials with SS analogs in the treatment of colorectal cancer resulted in only limited effects [10]. Thus, it is worth studying the various aspects of antitumoral actions of 5-FU and SS analogs.

The aim of the present paper was to compare the effects of two SS analogs: octreotide and lanreotide administered separately or together with 5-FU with the action of 5-FU alone, on proliferation, apoptosis, proliferation/apoptosis ratio and tumor weight in the murine transplantable Colon 38 cancer.

Materials and Methods

Experimental protocol

Two experiments were carried out on male mice of B6D2F1 strain, weighing 20±5 g. This strain is the first generation of the cross-breed between C57BL/6 and DBA/2 strains. The animals were subcutaneously (sc) treated with 33% suspension of Colon 38 cancer cells to induce tumors.

The first experiment

Eleven days after the induction of tumors, all the animals were divided into 4 groups (8-10 animals each) and treated with the following substances once daily for 6 days:
Group I: control, 0.2 ml of physiological saline, sc;
Group II: octreotide (SMS, Sandostatin, Novartis), 10 μg/animal, sc;
Group III: 5-fluorouracil (5-FU, Fluorouracil, Polpharma S. A., Poland) at a single dose of 70 mg/kg b.w. intraperitoneally (ip), on the second day of the experiment and in the rest days—physiological saline;
Group IV: SMS + 5-FU at the above doses.

Twenty-four hours after the last injection, all the animals were killed by spinal cord dislocation. Ninety minutes earlier all the mice received a single ip injection of bromodeoxyuridine (BrdU, Sigma) at a dose of 30 mg/kg b.w.

The second experiment

Seven days after the tumor implantation, the mice were divided into 4 groups and treated as follows:
Group I: control, received sc 0.2 ml of dimethyl sulphoxide at days 1, 4, 7 and 10 of the experiment;
Group II: lanreotide (LAN, Somatuline L.P., Ipsen Biotech) in a single injection, at a dose of 1 mg/animal on the first day of the experiment, intramuscularly (im);
Group III: 5-fluorouracil (5-FU, Fluorouracil, Polpharma S.A., Poland) at a dose of 70 mg/kg b.w. ip, on the second and eighth day of the experiment;
Group IV: LAN + 5-FU at the above doses. On the eleventh day, the animals were killed by spinal cord dislocation receiving 90 min earlier a single ip injection of BrdU (Sigma) at a dose of 50 mg/kg b.w.

In both experiments all detectable tumors were removed, weighed and fixed in 4% (v/v) formaldehyde (formalin) in PBS, and then embedded in paraffin wax. After paraffin-processing, serial 4–6 μm tissue sections were mounted on normal glass slides (for BrdU immunohistochemistry) or on aminopropyltriethoxysilane-treated Super Frost glass slides (for detecting apoptosis). In the microscopic preparations three parameters were assessed: the BrdU labeling index (LI), as an index of cell proliferation; the apoptotic index (AI), as an index of programmed cell death; and the proliferation/apoptosis (P/A) ratio.

Cell proliferation

The BrdU labeling index was the number of BrdU-immunopositive cell nuclei detected by immunostaining using the Cell Proliferation kit (Amersham), counted per 1000 randomly scored tumoral cell nuclei. Bromodeoxyuridine is a pyrimidine analog and like thymidine it is incorporated into DNA when cells enter the S phase of the cell cycle. Therefore, cells in the S phase can be identified

by immmunocytochemical staining with anti-BrdU monoclonal antibody [11]. It is a simple and repetitive morphological method used to measure cell proliferation. This method is more specific for our purposes than the stathmokinetic one (metaphase arrest), as it excludes the measurement of picnotic cells (stathmokinetic method) and is more simple than autoradiography with ^{3}H-thymidine.

Apoptosis

The apoptosis was visualized by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. The apoptotic index equal to the number of cells containing apoptotic bodies or nuclei assessed per 1000 randomly scored tumoral cell nuclei. Apoptotic cells were often identified in the compromised rim of necrotic zones, which typically exist in large tumors, and such areas were excluded from the assessment of AI. The laboratory protocol was adopted from Gavrieli et al. [12] and was performed with the reagent of In Situ Cell Death Detection Kit, POD (Boehringer Mannheim) according to the manufacturer's instructions with necessary modification to detect the apoptosis. Briefly, the tissue sections adhered to slides pretreated with silane, were heated for about 30 min at the temperature of 60°C to avoid section detachment and then deparaffinized and rehydrated. The slides were incubated for 15 min at room temperature in 20 μg/ml of proteinase K (Sigma) after preliminary tests consisting of varying concentration and incubation time. Other modifications of the kit were tested earlier and adjusted to the type of the examined tissues. We have used the reagents without the dilution. After the step with terminal deoxynucleotidyl transferase (TdT), the specimens were additionally saturated in 5% normal sheep serum to diminish the background. The color reaction with DAB (diaminobenzidine tetrahydrochloride) lasted for about 15 min. After that, the tissue sections were counterstained with haematoxylin for about 40 sec and mounted in Geltol Permanent, Aqueous Mounting Medium (Immunon). Negative control was performed by omitting TdT.

Statistical analysis

The data were analyzed statistically using the Mann-Whitney-Wilcoxon test. $P < 0.05$ was considered as the borderline of statistic significance.

Results

Tumor weight

The data concerning tumor weight are shown on Figure 1. Except for groups treated with SS analogs alone, all the treatment options resulted in more or less pronounced reduction of the mean tumor mass, but only in the group treated with 5-FU plus SMS a statistically significant difference vs. control group has been observed (means ± SEM; 75.6±37.7 vs. 434.8±114.8). The difference between tumor weight of the two control groups has not been estimated because the experimental protocols differ considerably in both studies. 5-FU alone did not decrease significantly the mean tumor weight at either of the two experiments (Exp. I: 5-FU-180.9±71.2 vs. C-434.8± 114.8; Exp. II: 5-FU-205.9±127.9 vs. C-286.8± 130.7). The same tendency concerning the tumor mass was observed in both experiments: the lowest mean (or median) tumor weight were shown in the group treated with 5-FU plus SMS or plus LAN and were lower than in the groups treated with 5-FU alone.

Cell proliferation (LI), apoptosis (AI) and proliferation/apoptosis (P/A) ratio

It has been found that both SS analogs given alone significantly decreased LI of Colon 38 tumors (SMS-127.6±19.1; LAN-169.1±11.3) (Figure 2), increased their AI (SMS-58.8±3.3; LAN-50.4±3.4) (Figure 3), and decreased P/A ratio (SMS-2.1±0.4; LAN-3.5±0.5) (Figure 4) as compared to control groups (Exp. I: LI-255.7±18.5; AI-18.0±1.1; P/A-15.1±2.1; Exp. II: LI-232.6±20.5; AI-26.5±2.9; P/A-9.1±0.9). 5-FU given alone significantly enhanced AI (Exp.I-60.3±9.9; Exp.II-62.3±7.1), decreased the P/A ratio (Exp.I-3.1±0.6; Exp.II-3.7±0.2) and did not change LI (Exp.I-187.6±39.1; Exp.II-233.2±30.0) of tumors as compared to controls (Figures 2, 3, 4). The joint treatment with 5-FU plus SMS or plus LAN caused a significant increase in AI (5-FU+SMS-56.1±4.1; 5-FU+LAN-67.3±5.4) and a decrease in P/A ratio (5-FU+SMS-3.1±0.3; 5-FU+LAN-3.2±0.7) but did not significantly change LI of tumors (5-FU+SMS-175.0±27.4; 5-FU+LAN-207.0±36.4) as compared to control groups (Figures 2, 3, 4). The joint action of 5-FU with one of two examined SS analogs on the LI, AI and P/A ratio did not differ statistically significantly from the effects of these substances applied alone, but it has to be stated that the observed inhibitory effects of both SS analogs on LI of the tumors were abolished by the addition of 5-FU (Figure 2). There

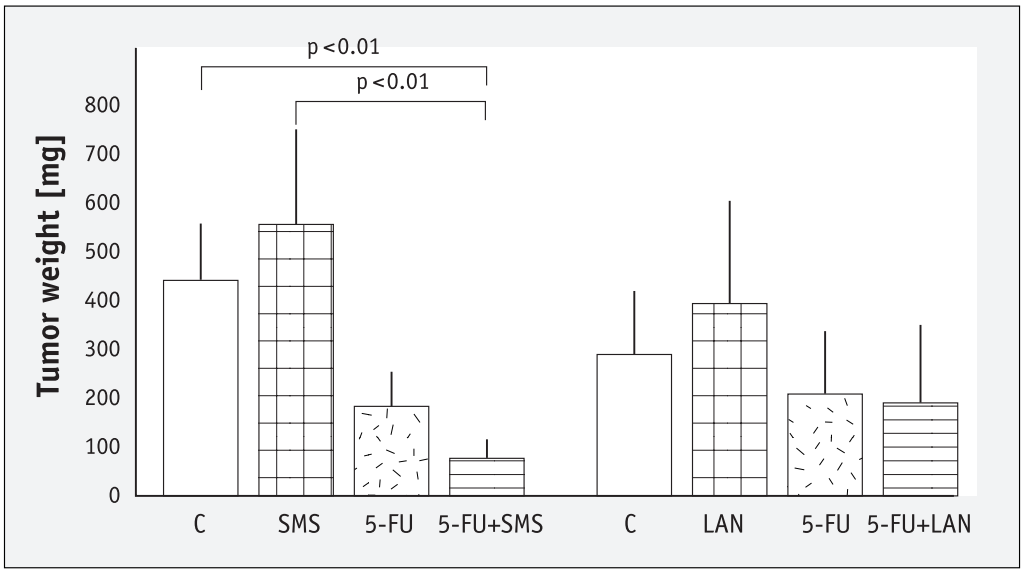

Fig. 1. Effects of SMS, LAN and 5-FU, alone or in combination, on Colon 38 tumor weight (mg) in mice. Bars present means ± SEM.

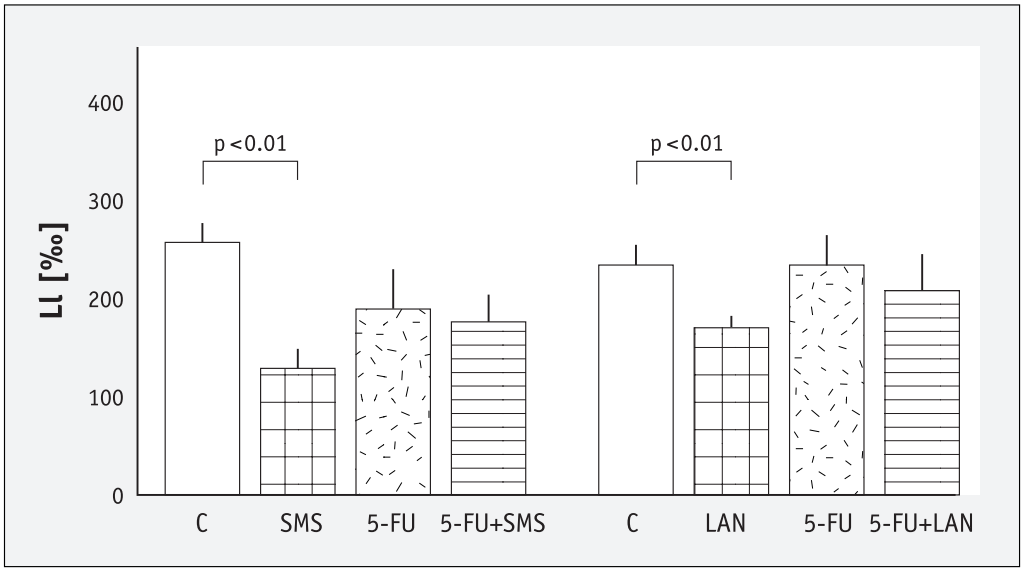

Fig. 2. Effects of SMS, LAN and 5-FU, alone or in combination, on BrDU labeling index (LI) in Colon 38 cancer cells. Bars present means ± SEM.

were neither additive nor synergistic effects between 5-FU and SS analogs on the LI, AI and P/A ratios.

Discussion

The data presented above show that the treatment with SS analogs, octreotide and lanreotide, exert an antiproliferative effect on Colon 38 tumor cells in vivo. These findings corroborate with earlier papers coming from our and other laboratories [4, 5, 6]. Unexpectedly, we did not find a decrease of BrdU-incorporation into tumor cell nuclei in the groups treated with 5-FU. This lack of effect may be connected with 5-FU dosage used in our study. We specially chose a relatively low dose of 5-FU, because we wanted to check the biomodulation effects of SS analogs on 5-FU action. The other authors showed that only the higher of two tested doses of 5-FU has been effective in the inhibition of ^{3}H-thymidine incorporation (^{3}H-thymidine like BrdU incorporates into cell nuclei during S phase) into colonic epithelium in mice [13] and in human colon cancer cell line [7]. It can be also taken under consideration that 5-FU may under certain conditions paradoxically enhance the incorporation of either ^{3}H-thymidine or BrdU into cell nuclei by decreasing the endogenous pool of thymidine caused by thymidylate synthase inhibition [14]. The thymidylate synthase is a critical target enzyme for 5-FU cytotoxic action. Thus, we decided to estimate the proliferative activity of the tumoral cells via another method. There was no decrease in the number of PCNA (Proliferating Cell Nuclear Antigen)-positive cells in the groups treated with 5-FU as compared to the controls (data not

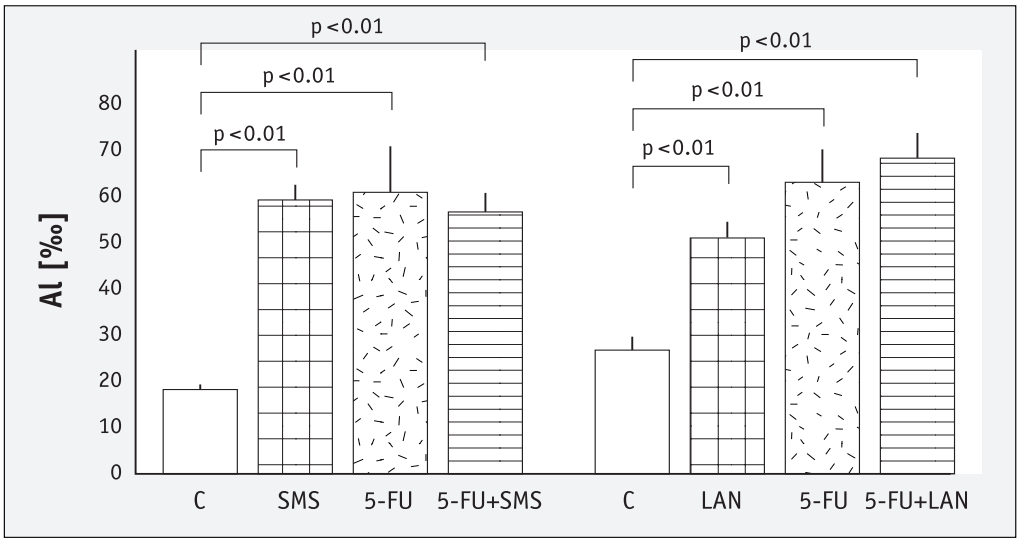

Fig. 3. Effects of SMS, LAN and 5-FU, alone or in combination, on apoptotic index (AI) in Colon 38 cancer cells. Bars present means ± SEM.

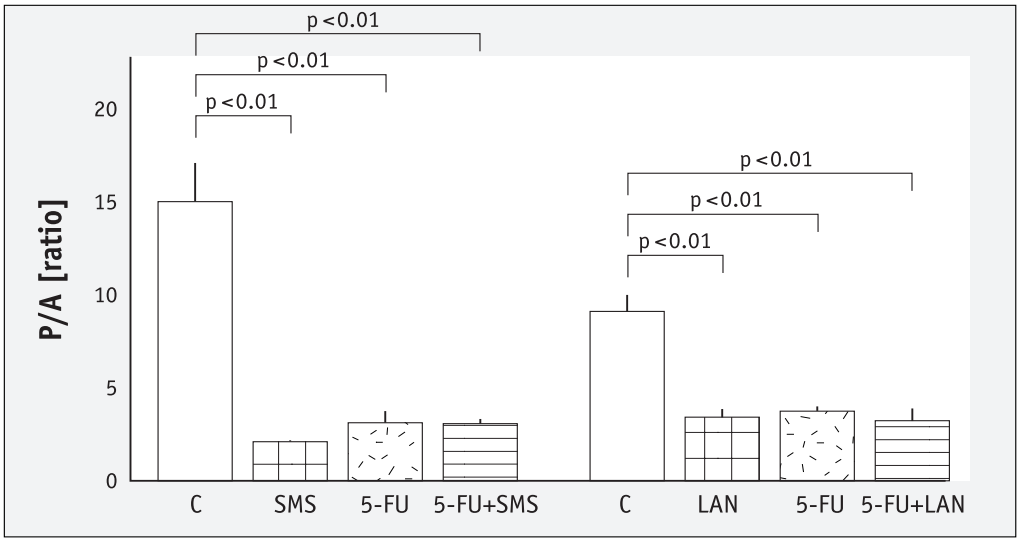

Fig. 4. Effects of SMS, LAN and 5-FU, alone or in combination, on proliferation/apoptosis ratio in Colon 38 cancer cells. Bars present means ± SEM.

shown). Our results do not seem to be related to the kind of the applied proliferation marker.

The joint treatment with 5-FU and each of the SS analogs reversed the inhibitory effect of both peptides on the proliferation of tumor cells. Our results are in contradiction to the study of Romani and Morris [7], who found that octreotide enhanced antiproliferative effect of 5-FU on the human colonic cancer cells, even in the case when 5-FU treated alone did not inhibit the proliferation. However, the cited study was performed in vitro and perhaps only in those conditions the synergistic effect between 5-FU and octreotide occurs. Some additive effect in vitro of both substances was reported also by Weckbecker et al. [15] on the pancreatic cancer cells proliferation. So far, there are no data about the interaction between 5-FU and octreotide on apoptosis in tumoral cells.

Either 5-FU or SS analogs were found to increase the apoptotic index of the investigated tumors. The proapoptotic effect of octreotide on Colon 38 tumor was shown also in our earlier paper [6]. The proapoptotic effect of 5-FU on gastrointestinal neoplasms was also demonstrated by other authors [16-18]. Both SS analogs used in our study are known to act preferentially via SS receptor subtype SSTR2, but may interact also with SSTR3 and SSTR5 [19]. The recent study of Sharma et al. [20] indicates that SS and its analogs induce apoptosis acting via SSTR3. This action is connected with the expression of wild type of tumor suppressor gene p-53 and bax. In the present study we did not reveal either additive or synergistic effects of 5-FU and SS analogs on apoptosis. The lack of additive or synergistic effects of 5-FU and SS analogs on apoptosis may be connected with common intracellu-

lar pathway in apoptosis induction by the investigated compounds. For instance, both SS analogs and 5-FU induce p-53 expression in target cells [13, 20, 21], and perhaps this common pathway did not allow evoking a synergistic effect of both substances on the apoptosis. Expression of p-53 is also involved in the apoptosis induced by the withdrawal of growth factors [22]. It is worth recalling that SS and its analogs inhibit growth factor secretion and/or antagonize their actions.

In spite of the significant alterations in proliferation and apoptosis indices in the investigated tumor, there were no significant changes (except the group treated with 5-FU plus SMS) in the tumor mass. This may result from the great dispersion of the individual values of tumor weight inside the experimental groups or from too short a duration of the experiments. Nevertheless, there was some tendency toward lower tumor weight in animals treated with 5-FU together with each of SS analog. It is unclear why the joint administration of 5-FU and SMS resulted in decreased tumor mass, taking into consideration that both substances had no additive effect on apoptosis and 5-FU even reverses the antiproliferative action of SMS. Possibly, neither proliferation nor apoptosis play a pivotal role in the joint antitumoral action of 5-FU and SMS. This effect depends rather on the antiangiogenic effect of SMS [23], which could reduce the tumor mass causing the necrotic changes.

Summing up, we found that both investigated SS analogs inhibit proliferation and induce apoptosis in Colon 38 tumor. On the other hand, we did not find either synergistic or additive effects of 5-FU and SS analogs on proliferation and apoptosis indices in the investigated colonic tumor. However, we cannot totally rule out such therapeutical synergy because of some alterations, albeit not always statistically significant, of the tumor mass.

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REFERENCES

1 Pawlikowski M, Kunert-Radek J, Stepién H. Somatostatin—an antiproliferative hormone. In: Döhler KD, Pawlikowski M, editors. Progress in Neuropeptide Research. Basel: Birkhauser Verlag; 1989. p. 3–12.
2 Schally AV. Oncological applications of somatostatin analogues. Cancer Res 1988; **48**:6977–85.
3 Reubi JC. Octreotide and nonendocrine tumors: basic knowledge and therapeutic potential. In: Lomax P, Scarpignato C, Vesell ES, editors. Progress in Basic and Clinical Pharmacology. Octreotide: from Basic Science to Clinical Medicine. Basel: Karger; 1996. p. 246–69.
4 Dy DY, Whitehead RH, Morris DL. SMS 201 995 inhibits in vitro and in vivo growth of human colon cancer. Cancer Res 1992; **52**:917–23.
5 Pawlikowski M, Kunert-Radek J, Winczyk K. Differential effects of somatostatin analogues on proliferation of murine colonic cancer cells in vitro. Cytobios 1997; **89**:183–7.
6 Melen-Mucha G, Winczyk K, Pawlikowski M. Somatostatin analogue octreotide and melatonin inhibit bromodeoxyuridine incorporation into cell nuclei and enhance apoptosis in the transplantable murine Colon 38 cancer. Anticancer Res 1998; **18**:3615–9.
7 Romani R, Morris DL. SMS 201.995 (Sandostatin) enhances in-vitro effects of 5-fluorouracil in colorectal cancer. Eur J Surg Oncol 1995; **21**:27–32.
8 Pawlikowski M, Winczyk K, Melen-Mucha G. Effect of somatostatin analog octreotide and some other antiproliferative agents on growth of transplantable colonic cancer in mice. Med Sci Monit 1997; **3**:465–7.
9 Chen TB, Huzak M, Macura S, Vuk-Pavlovi S. Somatostatin analogue octreotide modulates metabolism and effects of 5-fluorouracil and 5-fluorouridine in human colon cancer spheroids. Cancer Lett 1994; **86**:41–51.
10 Smith JP, Croitoru R, Doll D, Thornton C, Perry MC. Effects of octreotide, a long acting somatostatin analog, on advanced colon cancer. Gastroenterology 1992; **102**(suppl 4):A 399.
11 Gratzner HG. Monoclonal antibody to 5-bromo-and 5-iododeoxyuridine: a new reagent for detection of DNA replication. Science 1982; **218**:474–5.
12 Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. J Cell Biol 1992; **119**:493–501.
13 Pritchard DM, Potten CS, Hickman JA. The relationships between p53-dependent apoptosis, inhibition of proliferation and 5-fluorouracil-induced histopathology in murine intestinal epithelia. Cancer Res 1998; **58**:5453–65.
14 Greenberg HS, Chandler WF, Ensminger WD. Radiosensitization with carotid intra-arterial bromodeoxyuridine +/- 5-fluorouracil biomodulation for malignant gliomas. Neurology 1994; **44**:1715–20.
15 Weckbecker G, Raulf F, Tolcsvai L, Bruns C. Potentiation of the antiproliferative effects of anticancer drugs by octreotide in vitro and in vivo. Digestion 1995; **57**(suppl.1):22–8.
16 Inada T, Ichikawa A, Kubota T, Ogata Y, Moossa A, Hoffman RM. 5-FU-induced apoptosis correlates with efficacy against human gastric and colon cancer xenografts in nude mice. Anticancer Res 1997; **17**:1965–72.
17 Yamane N, Makino M, Kaibara N. S-phase accumulation precedes apoptosis induced by preoperative treatment with 5-fluorouracil in human colorectal carcinoma cells. Cancer 1999; **85**:309–17.
18 Sharma R, Adam E, Schumacher U. The action of 5-fluorouracil on human HT29 colon cancer cells grown in SCID mice: mitosis, apoptosis and cell differentiation. Br J Cancer 1997; **76(8)**:1011–16.
19 Morgan B, Coy D, Taylor J, Murphy W. The influence of structure on subtype selectivity and efficacy at somatostatin receptors. J Endocrinol Invest 1997; **20** (suppl to No. 7):5–7.
20 Sharma K, Patel YC, Srikant CB. Subtype-selective induction of wild-type p53 and apoptosis, but not cell cycle arrest, by human somatostatin receptor 3. Mol Endocrinol 1996; **10**:1688–96.
21 Brown JM, Wouters BG. Apoptosis, p53, and tumor cell sensitivity to anticancer agents. Cancer Res 1999; **59**:1391–99.
22 Canman CE, Gilmer TM, Counts SB, Kastan MB. Growth factor modulation of p53 mediated growth arrest versus apoptosis. Genes Develop 1995; **9**:600–11.
23 Danesi R, Del Tacca M. Effects of octreotide on angiogenesis. In: Lomax P, Scarpignato C, Vesell ES, editors. Progress in Basic and Clinical Pharmacology. Octreotide: from Basic Science to Clinical Medicine. Basel: Karger; 1996. p. 234–45.