

A comparison between double and triple therapies of octreotide, galanin and serotonin on a rat colon carcinoma

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Summary. Sixty female nude mice (C578L/6jBom-nu) were injected with 100 μ l cell suspension containing 2×10^6 viable cells of an N-methyl-N-nitroguanidine-induced rat colonic adenocarcinoma. After seven days the animals were divided into five groups. The first group received only saline and served as a control group. The second group received a triple therapy of octreotide, galanin and serotonin (20 μ g/kg). The last three groups received double therapies of octreotide/galanin, octreotide/serotonin or galanin/serotonin (20 μ g/kg). They were treated twice a day for five days. Tumour volume and weight, relative volume density of tumour-feeding blood vessels and of tumour necrotic tissue, as well as apoptotic and proliferation indices were determined. Animal weight, food consumption, faeces weight and its water content were recorded before and after treatment. Tumour volume was significantly reduced only in the group that received the triple therapy. The volume density of the tumour-feeding blood vessels was significantly reduced in the treated groups with the exception of the group that received octreotide and serotonin. Increased relative volume density of tumour necrotic tissue occurred only in the group treated with triple therapy. Apoptotic indices were significantly increased in all treated groups. No statistical difference was found between treated animals and controls regarding proliferation indices, food consumption, faeces weight and water content or animal weight. In conclusion, double therapy using two of the gastrointestinal bioactive substances, octreotide, galanin and serotonin, has certain effects on colon cancer cells. To cause a considerable tumour necrosis, triple therapy seems to be required. Both double and triple therapy seem to lack obvious side-effects.

Key words: Cancer, Colon, Galanin, Octreotide, Serotonin, Therapy

Introduction

Low levels of somatostatin and galanin and low densities of somatostatin and serotonin cells have been reported in patients with colon carcinoma (El-Salhy et al., 1988a,b). Furthermore, the number of somatostatin and serotonin cells in the colon has been found to be restored in patients with rectal carcinoma who received pre-operative radiotherapy (El-Salhy et al., 1988c). It is possible, therefore, to assume that these gut signal substances might be involved in the pathogenesis of colorectal carcinoma.

Recent studies from our group (El-Salhy and Sitohy, 2002; El-Salhy et al., unpublished data) showed that five days of triple therapy with octreotide, galanin and serotonin reduced the size of rat colonic adenocarcinoma xenografts to nude mice by about 50%. Increased tumour necrosis and apoptosis seem to cause this reduction (El-Salhy and Sitohy, 2002; El-Salhy et al., unpublished data). Single therapies with each of these gut signal substances did not show any effects on the same rat colonic adenocarcinoma. It has not been clear, however, whether the presence of all three substances is necessary for their action on colonic carcinoma seen previously. Thus, the present study was undertaken to investigate whether dual synergism between two of the previously mentioned gut signal substances is as effective as triple treatment on a rat colonic carcinoma.

Material and methods

Animals

Sixty female nude mice (C578L/6JBom-nu) with a mean body weight of 20.2 g were purchased from the Breeding and Research Centre in Denmark. Each mouse was caged individually in a laminar filtered airflow cabinet under pathogen-free conditions. The mice were kept under a constant temperature ($22 \pm 2^\circ$) and relative humidity ($55 \pm 5\%$) with 12 h dark/light cycles. They were fed on a standard pellet diet (R 34, lactamin,

Vadstena, Stockholm) and given water *ad libitum*. Aseptic techniques under laminar airflow were applied to all our experiments. The local committee on animal ethics at Umeå University approved the investigation.

Tumour implantation

Each animal was injected subcutaneously with 100 μ l cell suspension containing 2×10^6 viable cells of an N-methyl-N-nitroguanidine-induced rat colonic adenocarcinoma (provided kindly by Professor L.-O. Hafström, Department of Surgery, University Hospital, Umeå, Sweden). The suspension was injected in the lower left side of the mice back. The greatest and the least diameters were measured after seven days and the volume was calculated using the formula:

$$\text{Volume} = (\text{mean diameter})^3 \pi / 6$$

Treatment

The animals were divided into five groups of 12 each. The first group was injected with 100 μ l saline and served as a control group. The second group received 100 μ l saline solution containing 20 μ g/kg body weight octreotide (Sandostatin®, Novartis), galanin (synthetic human galanin, Sigma, Stockholm) and serotonin (5-hydroxytryptamine, oxalate salt, Sigma). The third group was injected with 100 μ l saline containing 20 μ g/kg body weight octreotide and galanin. The fourth group was injected with 100 μ l saline containing 20 μ g/kg body weight serotonin and galanin, and the fifth group was injected with 100 μ l saline containing 20 μ g/kg body weight serotonin and octreotide. Injections were administered subcutaneously every 12 hours for five days. The animals were then weighed and killed in a CO₂ chamber the following day. The tumours were dissected carefully and their diameters were measured and weights taken. Three tissue samples were taken from each tumour, two from each periphery and one from the middle. The samples were fixed overnight in 4-buffered paraformaldehyde, embedded in paraffin wax and cut at 5 μ m. The slides were then coded to avoid experimenter bias during quantification.

Food consumption, faeces weight and water content, and animal body weight

Food consumption, faeces weight and faeces water content were measured during the first three days after tumour implantation and during the first three days after treatment. Food consumed by each mouse was calculated as the difference between the weight of the food at the beginning and at end of the observation period. The faeces were collected, weighed and dried at 100 °C in an oven for three hours, then reweighed. Water content was calculated using the formula:

$$\text{Water content (\%)} = 100(1 - \text{faeces weight after drying} / \text{faeces weight before drying})$$

Volume density of tumour blood vessels and necrotic tissues

The sections were stained with haematoxylin-eosin. The relative volume densities of the blood vessels and the necrotic tissues were determined by the classical stereological point-counting method (Weibel and Elias, 1967; Weibel et al., 1969) as adapted to computerised image analysis (El-Salhy et al., 1997). Briefly, an automated standard sequence analysis operation was applied in which a regular 400-point lattice was superimposed on the frame containing the tissue. Points other than the tumour tissues were erased and the points covering the blood vessels or necrotic tissues were pointed out with the computer “mouse”; by clicking on the mouse, a series of blue points appeared. The ratios between the number of points laying on the blood vessels or the necrotic tissues, and the number of points laying on the tumours were set up automatically. A Quantimet 500 MC image processing and analysis system (Leica, Cambridge, UK) linked to an Olympus microscope type BX50 was used. The sum of 30 randomly chosen fields were measured (10 in each area from each tumour) by using an x10 objective. At this magnification, each pixel of the image corresponded to 0.83 μ m and each field in the monitor represented a tissue area of 0.17 mm².

Apoptosis

Apoptosis was detected *in situ* by using a kit (TA5411 from R&D Systems, Inc., Minneapolis, MN, USA). This kit detects the DNA fragmentation by incorporation of biotinylated nucleotides at 3' OH end of the DNA fragments, using terminal deoxynucleotidyl transferase (TdT). A streptavidin-conjugated horseradish peroxidase binds specifically to the biotinylated DNA fragments. Diaminobenzidine tetrahydrochloride (DAB) was used to detect the peroxidase in the sections. The sections were counter-stained by methyl green. The *in situ* detection was performed according to the protocol supplied by the manufacturer. The controls included nuclease-generated positive controls, unlabelled sample controls and labelled untreated sections from normal tissue (mouse colon). The labelled and unlabelled nuclei in 10 randomly chosen fields from each tumour were counted. Each field in the monitor of the Quantimet represented 0.009 mm² of the tumour. The apoptotic index was calculated using the formula (Kerr et al., 1999):

$$\text{Apoptotic index} = \text{positive labelled cells} / (\text{positive labelled cells} + \text{negative stained cells}) \times 100$$

Proliferation

The tumour sections were immunostained with the avidin-biotin-complex (ABC) method, as described earlier (El-Salhy and Suhr, 1996), to detect the proliferation. Briefly, the sections were immersed in

0.5% H_2O_2 in 50ml Tris-buffer, pH 7.6, for 10 minutes to inactivate endogenous peroxidase, and were treated with 1% bovine serum for another 10 minutes to occupy the non-specific binding sites. The tissues were then incubated overnight with proliferating cell nuclear antigen (PCNA) (monoclonal code number M0879, dilution 1:50, Dakopatts) as the primary antibody at room temperature. The sections were then incubated with biotinylated rabbit anti-mouse IgG (monoclonal, in dilution 1:200) for 30 minutes at room temperature. This was followed by incubation with avidin-biotin-peroxidase complex, in dilution 1:200, for another 30 minutes. The sections were then immersed in 50ml Tris-buffer containing 25mg diaminobenzidine tetrachloride and 10 μ l of H_2O_2 (30%) followed by light counter-staining in Mayer's haematoxylin.

Specificity controls were the same as those described previously (El-Salhy and Suhr, 1996) and included replacing the primary antibodies with non-immune serum and preincubation of the antisera for 24 h at 4° C with the corresponding antigen (75 μ g/ml diluted antibody). The labelled and unlabelled nuclei in 10 randomly chosen fields similar to those used in apoptosis were counted. The proliferation index was calculated by using the formula (Kerr et al., 1999):
 Proliferation index = positive labelled cells / (positive labelled cells + negative stained cells) $\times$ 100

Statistical analysis

When Bartlett's test for homogeneity of variance showed that the difference between SDs was not significant, a one-way analysis of variance (ANOVA) was performed with a Tukey-Kramer multiple comparison as a post-test. When Bartlett's test was significant, a Kruskal-Wallis non-parametric ANOVA test was used with Dunn's multiple comparison as a post-test. P values below 0.5 were considered significant. For comparison between the animal weights before and after cancer implantation, a paired t-test was used.

Results

Two animals died from the group that had received triple therapy. While dissection of the first animal did not show any macroscopic abnormalities, the second had colon carcinoma and metastasis to the regional mesenteric lymphatic nodes. In addition, two mice died in the group treated with octreotide and galanin. One had a swollen stomach and metastasis in the regional mesenteric lymphatic nodes, and the other had colon carcinoma and metastasis in the regional mesenteric lymphatic nodes. These animals were excluded from the statistical analysis.

Tumour volume and weight

There were significant differences ($P < 0.001$)

regarding tumour volume between the various groups of mice investigated (Fig. 1). Tumour volume was significantly decreased in the group treated by triple therapy containing octreotide, galanin and serotonin, as compared with controls ($P = 0.03$). There was no difference between any of the other groups and controls regarding tumour volume after treatment ($P = 0.1$). No statistical difference regarding tumour weight was found between any groups ($P = 0.8$) (Fig. 2).

Tumour blood vessel density

The relative volume density of tumour blood vessels was significantly different in various groups of mice ($P < 0.0001$). The blood vessel volume density was significantly decreased in all the groups treated with triple and double therapy, with the exception of the group that received octreotide and serotonin, where no statistical significance was found when compared with

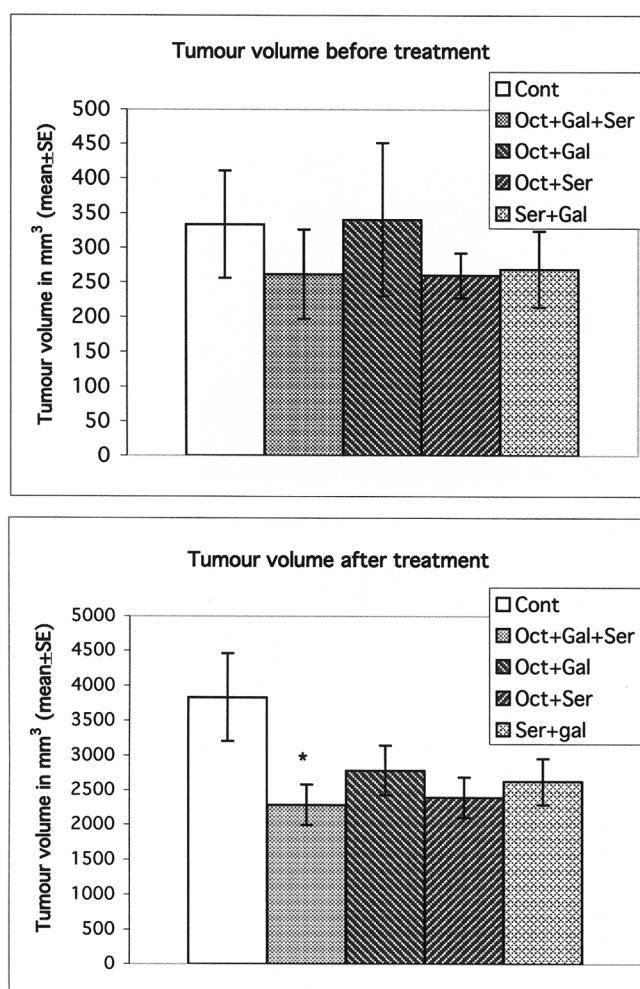


Fig. 1. The effect of triple and double therapy of octreotide, galanin and serotonin on the tumour volume. Cont: controls; Oct: octreotide; Gal: galanin; Ser: serotonin; *: $P < 0.05$.

Table 1. Faeces weight and water content in mice bearing colon carcinoma before and after treatment.

	FAECES WEIGHT (g)		FAECES WATER CONTENT (%)		FOOD CONSUMPTION (g)	
	Before	After	Before	After	Before	After
Controls	5.1±0.2	5.2±0.3	12.8±1	12.8±1	23.5±1.1	18.7±1
Oct±Gal±Ser	5.14±0.2	4.8±0.3	14.4±1.6	15.4±2	22.4±0.9	18.8±1.2
Oct±Gal	5.12±0.2	5.3±0.36	12.4±1.4	14.1±0.8	22.2±0.9	19.1±1.4
Oct±Ser	5±0.2	5.9±0.3	12.5±0.7	17.1±1.9	23.5±0.7	19.5±2
Ser±Gal	5.3±0.2	5±0.3	13.4±1	13±1.9	22.3±8.3	18.4±2.3

Oct: octreotide; Gal: galanin; Ser: serotonin

controls (Figs. 3, 4).

Apoptosis and proliferation

The apoptotic index was significantly different in various groups of mice ($P<0.0001$) (Figs. 5, 6). It was significantly increased in all treated groups. The proliferation index did not differ significantly between different mice groups ($P=0.6$) (Fig. 7).

Tumour necrotic tissue

The relative volume density of the tumour necrotic tissues was significantly different between the investigated groups of mice ($P=0.02$). It increased in all treated groups, but only statistically significantly in the group that had received triple therapy, as compared with controls (Fig. 8).

Food consumption, faeces weight and water content, and animal weight

The results of the measurements of food consumption, faeces weight and water content, as well as animal weight, are presented in Table 1 and Fig. 9. There

was no statistical difference between animal groups before or after treatment with either double or triple therapy regarding food consumption, faeces weight or faeces water content (before: $P=0.8$, 0.8 , 0.4 , respectively; and after: $P=0.6$, 0.2 , 0.3 , respectively). Animal weights of the different experimental groups did not show any statistical difference before or after treatment ($P=0.2$, 0.9 , respectively). However, there was a significant decrease in animal body weight after tumour implantation in controls and double therapy groups ($P=0.002$, 0.01 , 0.01 and 0.002 , respectively). The group treated with octreotide, galanin and serotonin did not show any significant difference in body weight before and after treatment ($P=0.5$).

Discussion

The present findings after triple therapy with octreotide, galanin and serotonin on a rat colon carcinoma are in line with earlier observations (El-Salhy and Sitohy, 2002; El-Salhy et al., unpublished data). Thus, triple therapy reduces the volume of the tumour, increases apoptosis and necrosis of the tumour and decreases tumour-feeding blood vessels. Similar to previous observations (El-Salhy and Sitohy, 2002), the tumour weight in animals received triple therapy did not decrease significantly from controls, despite reduced tumour volume. The tumour in these animals consisted of about 40% necrotic tissues. One may speculate that

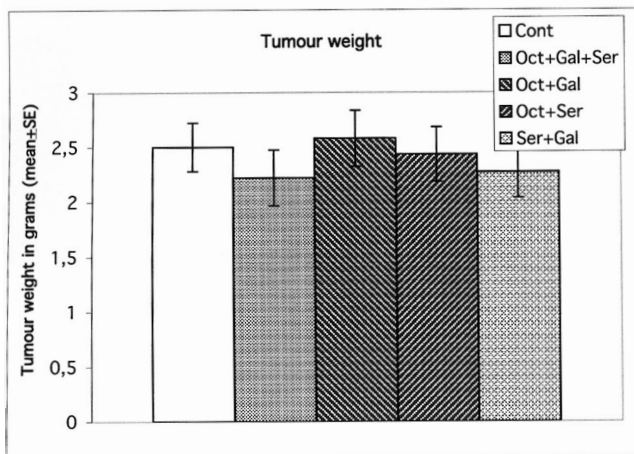


Fig. 2. The effect of triple and double therapy of octreotide, galanin and serotonin on the tumour weight. Symbols are the same as in Fig. 1.

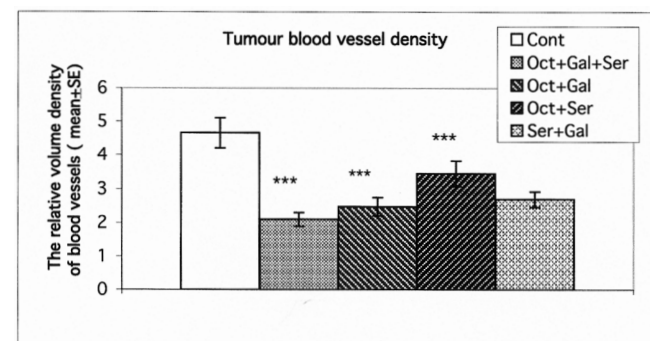


Fig. 3. The relative volume density (%) of tumour-feeding blood vessels after triple and double therapy with octreotide, galanin, and serotonin. Symbols are the same as in Fig. 1; ***: $P<0.001$.

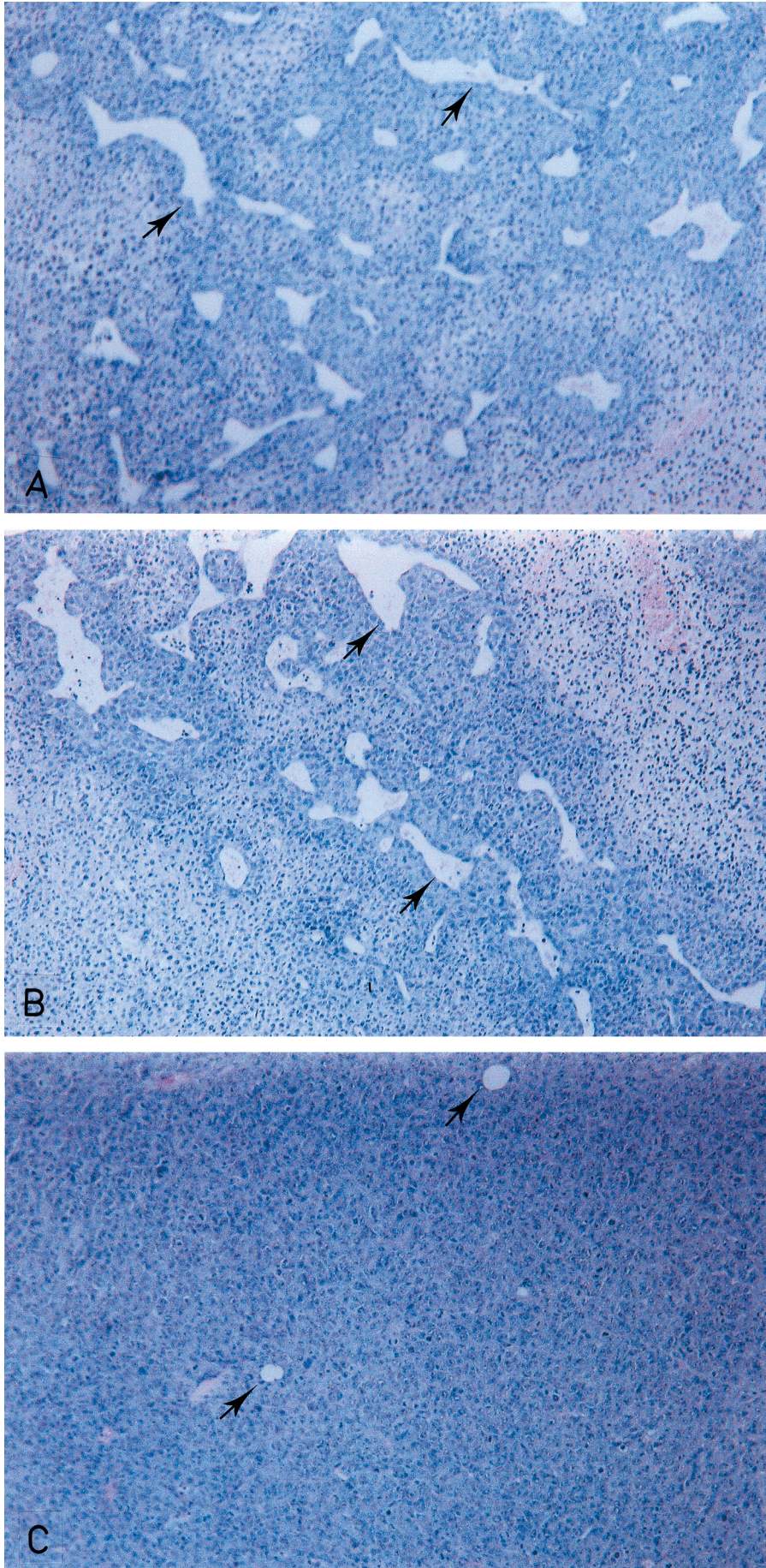
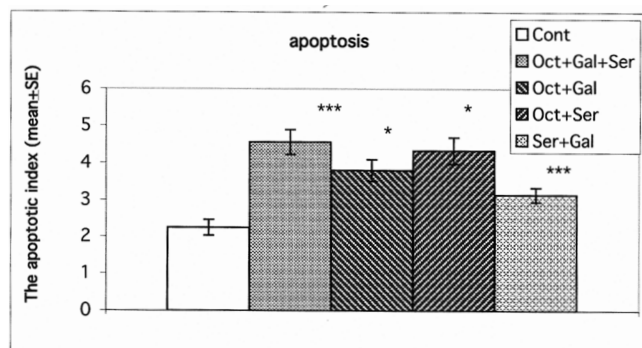


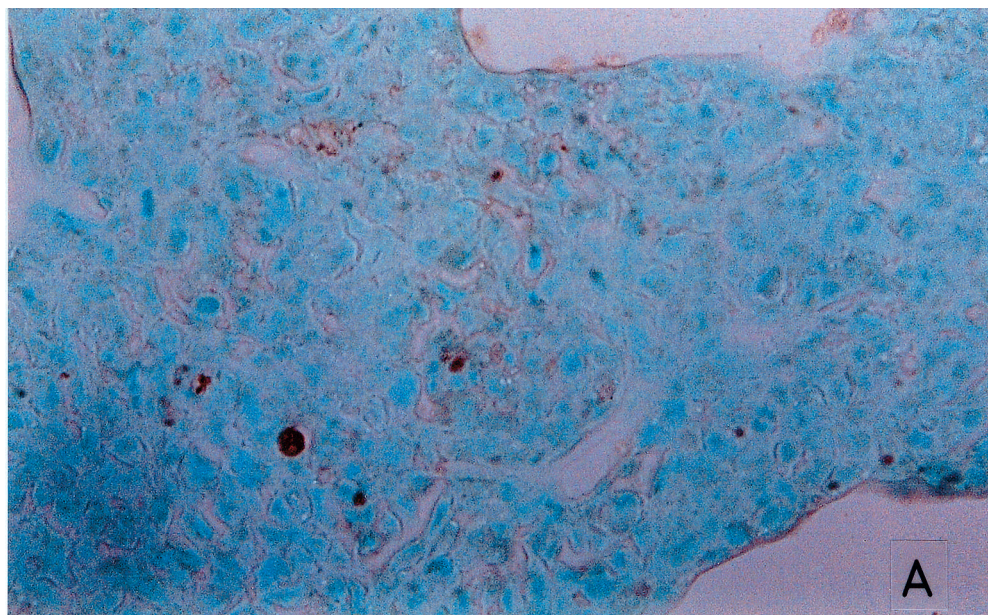
Fig. 4. The tumour-feeding blood vessels in a control (**A**), in a tumour treated with octreotide and serotonin (**B**) and in a tumour treated with galanin and serotonin (**C**). H&E. x 125



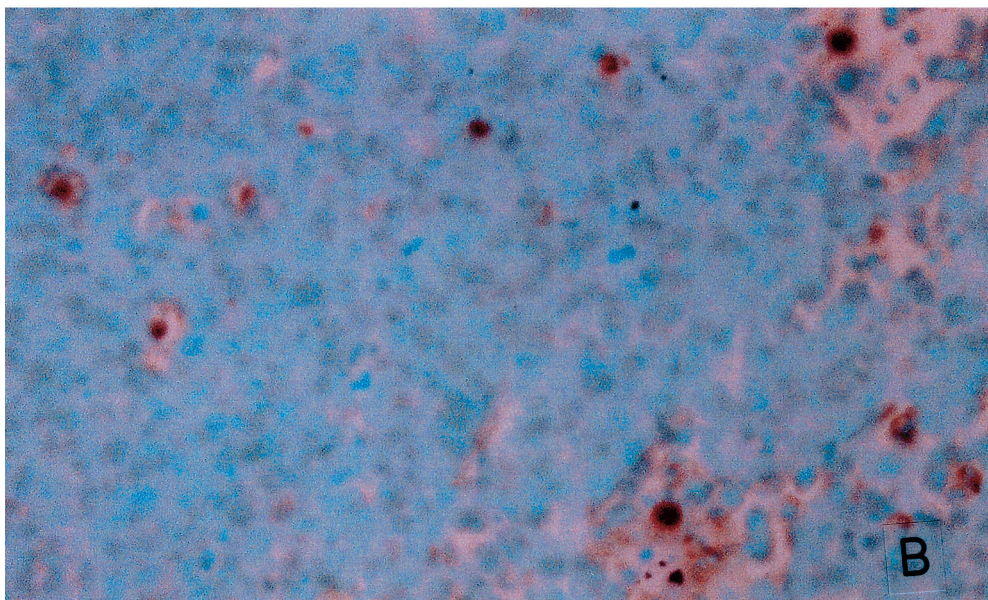
the necrotic tissue affects the architecture of the tumour and consequently the reduced volume but not the weight of the tumour.

Double therapy with any two of the bioactive gut substances (octreotide, galanin and serotonin) significantly increased apoptosis of the rat colon cancer cells. Somatostatin and somatostatin analogues in sufficiently high doses have been found to induce colon

Fig. 5. The apoptotic index in tumours received triple and double therapy with octreotide, galanin and serotonin Symbols are the same as Figs. 1 and 3.



A



B

Fig. 6. Apoptotic cells (brown) in a control (**A**) and in a tumour treated with serotonin and galanin (**B**). x 400

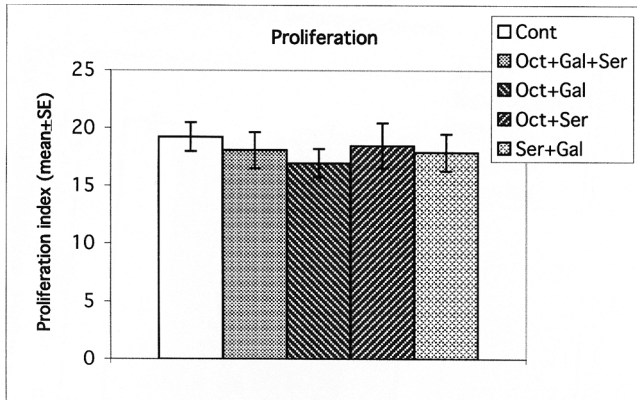


Fig. 7. The proliferation index in tumours treated with triple and double therapy of octreotide, galanin and serotonin. Symbols are the same as in Fig. 1.

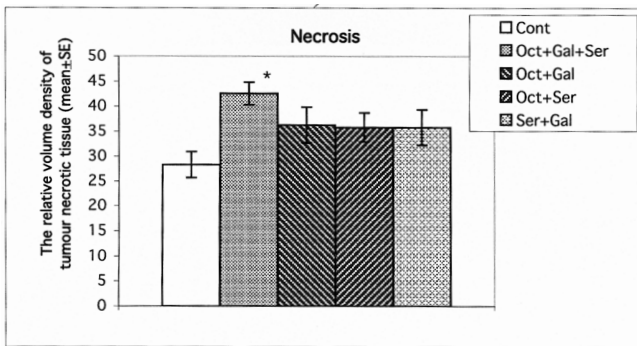


Fig. 8. The relative volume density of necrotic tissue in tumours received triple double therapy with octreotide, galanin and serotonin. Symbols are the same as in Fig. 1.

cancer cell apoptosis (Ferjou et al., 2002; Melen-Mucha et al., 2002). The increase in rat colon cancer cell apoptosis observed in double therapy in the present study cannot only be attributed to octreotide, as double therapy with galanin and serotonin also increased apoptosis. On the other hand, the reduced density of tumour-feeding blood vessels seen after double therapy is most probably caused by galanin, as suggested earlier (El-Salhy et al., unpublished data). This is supported by the finding in the present study that double therapy with octreotide and serotonin did not affect the density of the tumour-feeding blood vessels. It seems, however, that triple therapy with all the three gut bioactive substances is needed to induce a significant necrosis of the rat colon cancer studied.

Neither double therapy nor triple therapy of octreotide, galanin and serotonin appear to have marked side effects. Thus, animal weight and appetite have not been affected by the treatment. Moreover, the animals did not show any signs of diarrhoea or constipation. This is an important aspect for its probable clinical use in the

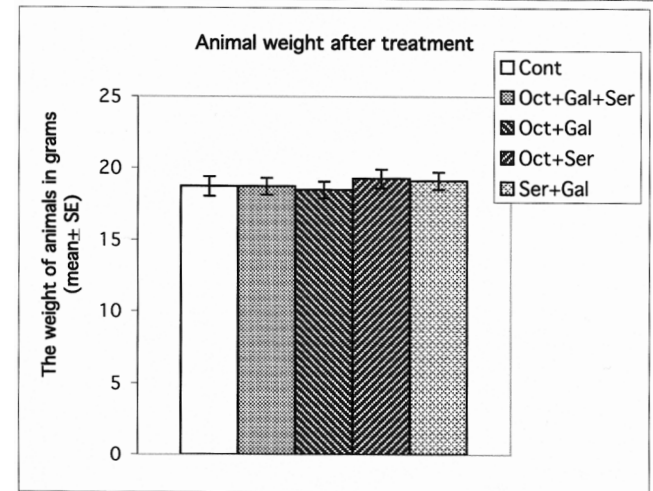
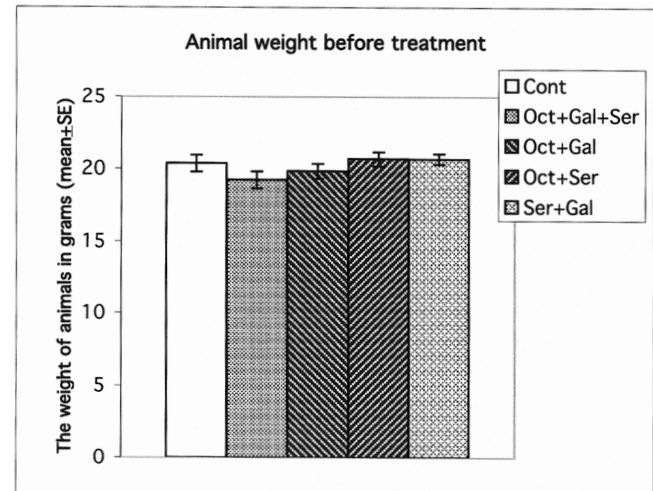


Fig. 9. The animal body weight before and after treatment. Symbols are the same as in Fig. 1.

future.

In conclusion, double therapy by two of the gut bioactive substances, octreotide, galanin and serotonin, has certain effects on rat colon cancer cells. Triple therapy is, however, required to bring this tumour to necrosis. Both double and triple therapy with these bioactive substances seem to lack obvious side effects.

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