

# Effects of single, double or triple combinations of octreotide, galanin and serotonin on a human pancreatic cancer cell line

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**Summary.** The human pancreatic cancer cell line (SW 1990) was exposed to 0.2 µg/ml of octreotide, galanin or serotonin as single, double or triple combinations. The tumor cells were checked at 3, 6 and 12 hours. In order to determine the number of viable cancer cells, the MTT-assay was used. Proliferation, apoptosis and the expression of epidermal growth factor were detected with immunohistochemistry using the avidin-biotin complex method. In addition, apoptosis was also detected with (TUNEL) method. The primary antibodies used were proliferating cell nuclear antigen (PCNA), anti-poly (ADP-ribose) polymerase (PARP) and anti-human epidermal growth factor. Single treatment with octreotide or serotonin reduced, the number of viable cells and the proliferation index at all observation times. Galanin increased the number of viable cells and the proliferation index. Whereas double treatments containing octreotide reduced the number of viable cells, those containing galanin increased the number. The effect of single, double or triple treatment on the apoptotic index obtained with both TUNEL method and PARP expression varied depending on the combination and the observation time. Octreotide did not affect the tumor cell expression of EGF. Galanin and serotonin, on the other hand, increased the expression of EGF. Whereas triple combination increased the expression of EGF after 6 h, all the other double combinations decreased this expression. It has been concluded that treatment with a combination of octreotide and serotonin may be useful in clinical settings.

**Key words:** Galanin, Octreotide, Pancreatic cancer, Serotonin

## Introduction

Pancreatic cancer is a common gastrointestinal malignancy with an exceptionally poor prognosis, where the mortality rate nearly matches the rate of incidence (Linder, 1997; Lowenfels and Maisonneuve, 2002). The only curative treatment is surgery, but only a minority (10-15%) of the patients have tumors amenable to surgical resection (Sohn et al., 2000; Friess et al., 2003).

It has been shown that triple therapy with three signal substances naturally occurring in the colon (somatostatin, galanin and serotonin) considerably reduced the volume and weight of human and rat colon carcinoma in xenografts (El-Salhy and Sitohy, 2002; El-Salhy et al., 2003; Sitohy and El-Salhy, 2003; El-Salhy, 2004, 2005). This reduction was attributed to increased apoptosis and decreased vascularization and proliferation of the tumor cells (El-Salhy and Sitohy, 2002; El-Salhy et al., 2003; El-Salhy, 2004, 2005; Sitohy and El-Salhy, 2003). Comparison of triple therapy and standard chemotherapy, namely 5-fluorouracil/leucovorin (5-FU/LV), showed that triple therapy has the same anti-tumor effect as 5-FU/LV but better therapeutic efficacy (El-Salhy, 2004a). It appears that this form of treatment is free from side effects (El-Salhy and Sitohy, 2002; Sitohy and El-Salhy, 2003).

In contrast to the previous findings in colon cancer (El-Salhy and Sitohy, 2002; El-Salhy et al., 2003; Sitohy and El-Salhy, 2003; El-Salhy, 2004a,b), triple therapy with octreotide, galanin and serotonin has been found to increase the proliferation and expression of epidermal growth factor (EGF) of pancreatic cancer cells (El-Salhy et al., 2004). It is unclear, however, whether this increase in proliferation and expression of EGF is caused by one, two or by all three of these bioactive substances. The present study was therefore undertaken to clarify this matter.

## Material and methods

### *Cell line and culture conditions*

The human pancreatic adenocarcinoma cell line SW

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1990 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Leibovitz L15 medium (Sigma-Aldrich, Stockholm, Sweden) supplemented with 10% (v/v) fetal bovine serum, L-Glutamine, penicillin and streptomycin (Sigma). Adherent monolayer cultures were maintained on plastic at 37 °C in a humidified incubator without CO<sub>2</sub>. Cells were detached using trypsin-EDTA and passed by into a new 75-cm<sup>2</sup> flask once a week.

#### *Treatment with octreotide, galanin and serotonin*

The cells were detached with trypsin-EDTA and suspended in Leibovitz L15 medium with 10% fetal bovine serum, L-Glutamine, penicillin and streptomycin. From this cell suspension, 200 µl containing 5x10<sup>4</sup> viable cells were plated out on 96-well plates. The cells were allowed to attach and recover over night, then incubated with an additional 100 µl medium containing single, duple or triple combinations of octreotide (Sandostatin, Novartis), galanin (synthetic human galanin, Neosystems, Strasbourg, France) or serotonin (5-hydroxytryptamine, oxalate salt, Sigma). The final concentration of octreotide, galanin and serotonin, the pancreatic cancer cells was exposed to was 0.2 µg/ml. The choice of this concentration was based on earlier studies (El-Salhy and Starefeldt, 2003), where 0.2 µg/ml was calculated to correspond to 20 µg/kg body weight. The cells were incubated for 3, 6, and 12 h. In each experiment a control consisting of only complete medium was included.

#### *MTT-assay*

The MTT cell assay kit (Sigma) was used. The assay is based on the conversion of tetrazolium salt into blue formazan products by mitochondria during cell respiration. At the end of the treatment, the plates were removed from the incubator into laminar flow hood where the medium was replaced with 100 µl fresh medium and 10 µl tetrazodim salt and returned to the incubator for 4 hours, followed by 100 µl MTT solvent. The plates were left overnight in the dark at room temperature. They were then measured in an ELISA plate reader (Labdesign AB, Täby, Sweden) at 570 and 690 nm for subtracting background absorbance. The number of viable cells was calculated from a standard curve prepared from serial dilutions from 1x10<sup>6</sup> to 6.25x10<sup>4</sup> cells/ml.

#### *Cell preparation for morphological studies*

At the end of the experiments, the cells were detached with trypsin-EDTA and suspended in 1 ml of the culturing medium. Thermo Shandon Cytospin 4 Cytocentrifuge (Labex, Helsingborg, Sweden) was used to attach the cells uniformly to slide. The cells were processed according to the manufacturers instructions. Briefly, 150 µl cell suspension was centrifuged at 35.8xg

for 5 min and the following next 2 rounds were done with respectively 100 and 75 µl cell suspension without switching the filters. The cells were air-dried for 10 min and fixated in 4% buffered formaldehyde for another 10 min. The slides were rinsed in a 10% sucrose solution (w/v) in Tris buffer (pH 7.6), air-dried and stored at -70 °C until the time for immunostaining.

#### *Immunohistochemical staining*

The slides were thawed at room temperature and air-dried in a heating cabinet (37°C) for 1 hour. For immunostaining, the avidin-biotin complex (ABC) method was used (DakoCytomation, Glostrup, Denmark) as described earlier in detail (El-Salhy et al., 1998). The primary antibodies used were anti-proliferating cell nuclear antigen (PCNA) (monoclonal, code number M0879, diluted 1:50, Dako Cytomation), anti-human epidermal growth factor (monoclonal, EGF-10, diluted 1:1000, MAB 293, R&D Systems, Minneapolis, USA) and anti-poly (ADP-ribose) polymerase (PARP) (monoclonal, code number P248, diluted, 1:500, Sigma). The slides were pre-incubated with Proteinase K (DakoCytomation) for 4 minutes except for PCNA. The slides were slightly counterstained with haematoxylin.

#### *Terminal deoxynucleotidyl transferase-mediated end labeling (TUNEL) method*

To detect apoptosis the TACS•XL- Basic *in situ* apoptotic detection kit (R&D System Inc., Minneapolis, MN, USA) was used according to the protocol supplied by the manufacturer. This technique is based on detecting the DNA fragmentations characteristic for apoptotic cells. The fragmentations in the DNA are detected *in situ* by incorporating labeled nucleotides onto the free 3' OH ends of the fragments using terminal deoxynucleotidyl transferase enzyme (TdT) followed by detection of the labeled molecules.

#### *Morphometry*

Quantification was performed with the Quantimet 500MC image processing analysis system (Leica Cambridge, UK) linked to an Olympus microscope, type BX50 as described earlier (El-Salhy and Dennerqvist, 2004). The software used in this system is QWIN standard (Leica's Windows based image analysis tool kit, version 2.8). In addition, the system included QUIPS (version 2.8), an interactive programming system. The slides were coded and the performer was not aware of the identity of the slides. Cell counting was performed in 10 randomly chosen fields in each control and treated groups, using a 20X objective. At this magnification each pixel on the computer screen corresponded to 0.173 µm and the area of each field corresponded to 0.090 mm<sup>2</sup>. Proliferating, apoptotic and labeling indices were calculated as described previously (Kerr et al., 1999) as

follows:

The labeling index= (the number of labeled cells / total number of cells) x100.

## Results

### Viable cells

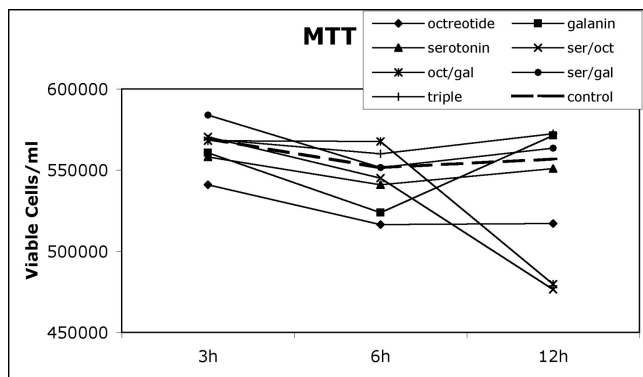
The results of the MTT-assay are illustrated in Fig 1. Single treatment with octreotide reduced the number of viable cells at all observation times. Slight decrease in the number of viable cells was seen with single exposure to serotonin. Galanin reduced the number of viable cells initially after 6 h, but this number increased after 12h. Whereas double treatments containing octreotide reduced the number of viable cells, those containing

galanin increased the number of viable cells. Triple treatment increased the number of viable cells.

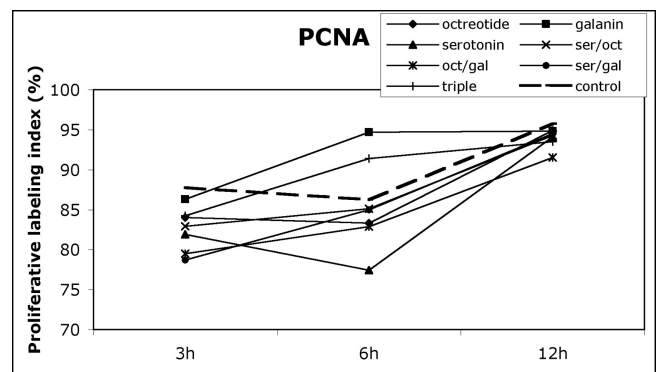
### Proliferation and apoptotic indices

Whereas octreotide and serotonin reduced proliferation index, the exposure to galanin increased this index. All the double as well as the triple combinations reduced the proliferation index in different degrees and at various intervals (Figs. 2, 3).

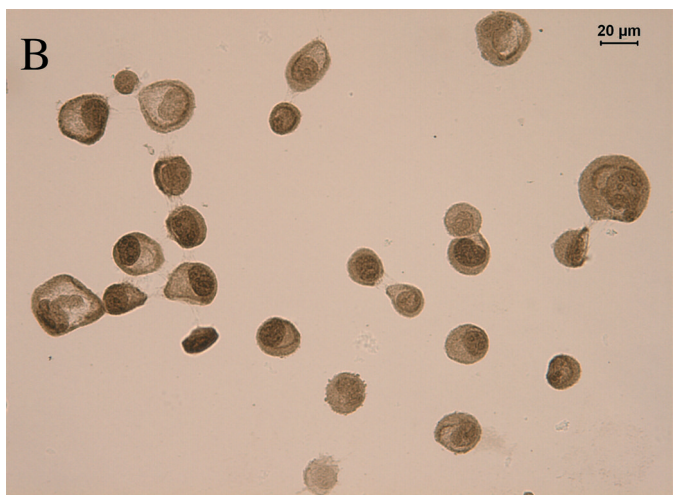
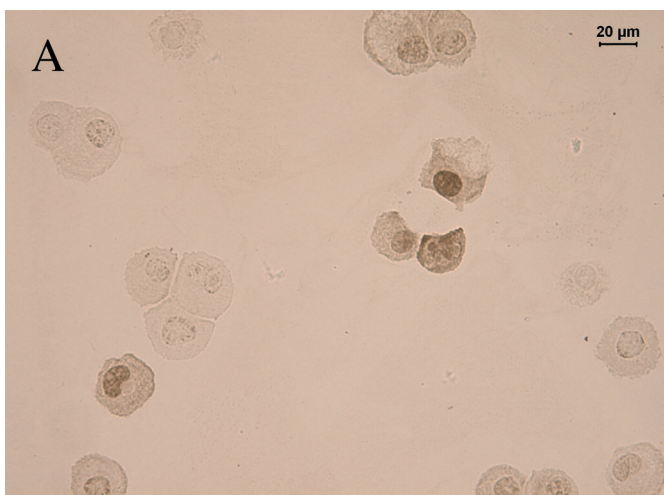
The effect of single, double and triple treatment on the apoptotic index obtained with both TUNEL method and PARP expression varied depending on the combination and the observation time (Figs. 4, 5). The combination of octreotide with either galanin or serotonin increased the apoptotic index at all observation



**Fig. 1.** Number of viable cells in controls and after incubation with octreotide, galanin and serotonin alone, in double or triple combinations. oct: octreotide; gal: galanin; ser: serotonin.



**Fig. 2.** Proliferation index in controls and following the incubation with octreotide, galanin and serotonin as single agents or in double or triple combinations. Symbols are the same as in Fig. 1.



**Fig. 3.** PCNA-immunoreactive cells in controls (A) and in tumour cells after 6 h treatment with galanin (B). The proliferating nuclei are stained brown.

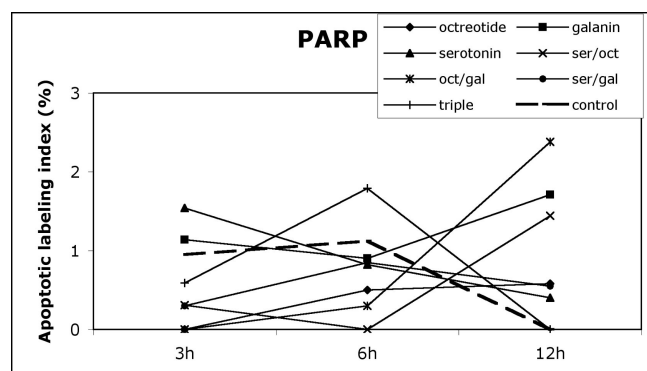


Fig. 4. Apoptotic index as revealed by PARP expression. Symbols are the same as in Fig. 1.

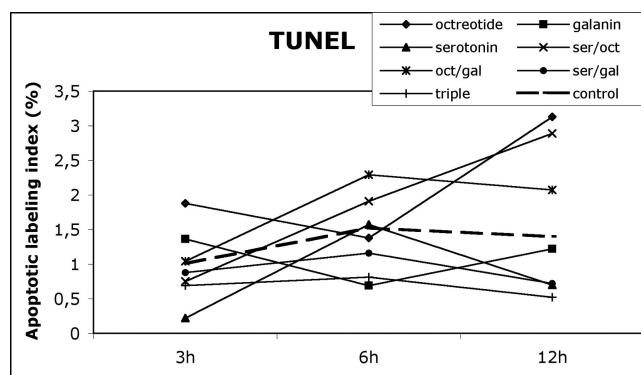


Fig. 5. Apoptotic index as detected by TUNEL method. Symbols are the same as in Fig. 1.

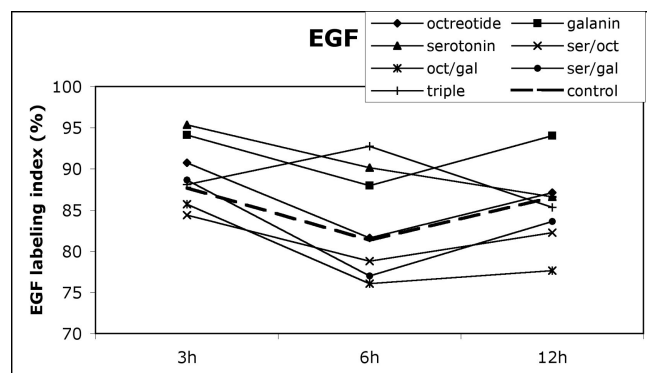


Fig. 6. EGF-labeling index in controls and treated pancreatic cancer cells. Symbols are the same as in Fig. 1.

times.

### Epidermal growth factor

Octreotide did not effect the expression of EGF of the tumor cells. Galanin and serotonin, on the other hand, increased the expression of EGF. Whereas triple combination increased the expression after 6 h, all the other double combinations decreased this expression (Fig. 6).

### Discussion

The present findings that octreotide reduces the number of viable cells and proliferation of pancreatic cancer cells are in agreement with previous *in vitro* and *in vivo* studies (Weckbecker et al., 1993; Kikutsuji et al., 2000; Hejna et al., 2002). Moreover, clinical trials have shown that treatment with octreotide improves the quality of life and prolongs survival in patients with non-

resectable ductal pancreatic adenocarcinoma (Wenger et al., 2001).

The present study showed that galanin and combinations containing galanin increased the proliferation and the expression of EGF of pancreatic cancer cells. This observation may explain the earlier findings *in vivo* with the same cell line, where triple treatment including galanin increased the cell proliferation and the expression of EGF. The present study showed further that this effect is caused by direct exposure of pancreatic cancer cells to galanin and not indirectly through changes in the host animal.

The effect of serotonin on pancreatic cancer is, to the best of our knowledge, not known. In the present study serotonin was found to decrease both the number of viable cells and the proliferation of the pancreatic cancer cell line. As single therapy with octreotide in patients with unresectable pancreatic cancer improves the quality of life and prolongs survival (Wenger et al., 2001), treatment with a combination of octreotide and serotonin may be useful in clinical settings.

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