Luzindole but not 4-phenyl-2-propionamidotetralin (4P-PDOT) diminishes the inhibitory effect of melatonin on murine Colon 38 cancer growth *in vitro*

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Abstract

OBJECTIVE: Our earlier studies have shown that MLT exerts the inhibitory effect on murine cancer via membrane and nuclear receptors. We have found that the antagonist of MT_1 receptors does not diminish the antiproliferative effect of MLT on Colon 38 cells, and the contribution of MT_2 receptors has been suggested to be responsible. Therefore, in the present study we have examined the influence of the 4-phenyl-2-propionamidotetralin (4P-PDOT), which is a selective antagonist of MT_2 membrane receptor, and luzindole – an antagonist of both membrane receptors, on an oncostatic action of MLT.

MATERIALS AND METHODS: The murine cancer cell line Colon 38 was used in the experiments. In 48 hrs cell culture the effects of MLT, 4P-PDOT and luzindole administered alone and MLT applied jointly with either 4P-PDOT or luzindole were examined. The growth of cancer cells was assessed using the modified colorimetric Mosmann method.

RESULTS: Melatonin at both examined concentrations (10^{-7}, 10^{-9} M) significantly decreased the viability of cancer cells. The selective antagonist of MT2 membrane receptor, namely 4P-PDOT and luzindole applied separately did not have an effect on the growth of Colon 38 cells. The addition of 4P-PDOT to MLT did not change the inhibitory effect of MLT, whereas luzindole given together with MLT diminished, but failed to block totally, the oncostatic properties of MLT.

CONCLUSIONS: The obtained data and our previous studies conducted on Colon 38 cancer indicate that membrane melatonin receptors are not indispensable to the oncostatic action of melatonin and thus other pathways such as nuclear signaling and receptor-independent mechanism may be also involved.

ORIGINAL ARTICLE

INTRODUCTION

The influence of melatonin (MLT) on the growth of various experimental cancers, including colon cancer, has been investigated intensively over recent years (Pawlikowski *et al.* 2002). It has been shown that MLT decreases the incidence, number and size of 1,2-dimethylhydrazine – induced colon tumors in rodents by suppressing tumor growth and invasiveness (Anisimov *et al.* 1997; 2000). The inhibitory effect of MLT on animal and human colon cancer cells in culture has been also reported (Farriol *et al.* 2000; García-Navarro *et al.* 2007). The majority of the investigations were related to the antiproliferative action of melatonin. Our previous study conducted on murine Colon 38 cancer showed that MLT inhibits cell proliferation *in vivo* and *in vitro* conditions (Melen-Mucha *et al.* 1998; Winczyk *et al.* 2002). Melatonin exerts the oncostatic effect also via induction of apoptosis. We have found that MLT enhanced the cells apoptosis in murine colonic cancer (Melen-Mucha *et al.* 1998; Winczyk *et al.* 2001). The proapoptotic effect of the pineal hormone was confirmed by other study (García-Santos *et al.* 2006; Martín-Renedo *et al.* 2008). Although data supporting the inhibitory action of MLT on the growth of human cancer is documented well enough in the literature, the molecular and cellular mechanisms by which this hormone can exert the oncostatic effect still remain unclear. Melatonin acts via the modulation of endocrine and immune systems and the said hormone may also directly influence cancer cells through specific binding sites. The best characterized binding sites of MLT are G protein-coupled membrane receptors, named MT_1 and MT_2 (Dubocovich *et al.* 1998). In mammals membrane receptors participate in the regulation of circadian and seasonal rhythms. The binding sites of melatonin were found also in gastrointenstinal tract, where this hormone plays an important role (Lee and Pang, 1993; Bubenik, 2008). As a small lipophilic molecule, melatonin easily crosses cellular membranes and may exert its biological effects through cytoplasmic and nuclear signaling. The nuclear orphan receptors named RZR/ROR were proposed as a nuclear binding sites for melatonin (Calberg and Wiesenberg, 1995; Wiesenber, 1998). The oncostatic effects of the pineal hormone seem to depend on membrane and nuclear receptors. It has been shown that melatonin-induced suppression of hepatoma growth is mediated via G-protein connected membrane receptors (Blask *et al.* 1999). The involvement of membrane receptors in antiproliferative effects of melatonin have been described in the following cell lines of human carcinoma: Jar and JEG-3 choriocarcinoma, MCF-7 breast cancer, Ishikawa endometrial cancer, LNcaP and Du-145 prostate cancers (Shiu *et al.* 1999; 2000; Ram *et al.* 2002; Kanishi *et al.* 2000; Xi *et al.* 2001; Marelli *et al.* 2000). The possibility of melatonin action via nuclear biding sites were examined in the human breast cancer, androgen-dependent and androgen-independent prostate cancers, the cells of which possess the RZR/ROR receptors (Dai *et al.* 2001; Moretti *et al.* 2000; 2001a). We have documented that both MT_1 and MT_2 membrane receptors as well as RZR/RORα receptors are expressed in Colon 38 cancer cells (Karasek *et al.* 2002). Our earlier study showed that melatonin exerts an inhibitory effect on murine cancer via membrane and nuclear receptors (Winczyk *et al.* 2002; Karasek *et al.* 1998). Moreover, we have found that antagonist of MT_1 receptors does not diminish the antiproliferative effect of the pineal hormone on Colon 38 cells, and the contribution of MT_2 receptors has been suggested. Therefore, in the present study we examined the influence of the 4-phenyl-2-propionamidotetralin (4P-PDOT) – a selective antagonist of MT_2 membrane receptor – as well as luzindole – an antagonist of MT_1 and MT_2 membrane receptors – on an oncostatic action of melatonin.

MATERIALS AND METHODS

Compounds

The following substances were examined in this study: melatonin (N-acetylo-5-metoksytryptamina, Sigma), 4-phenylo-2-propionamidotetralin (4-P-PDOT, Tocris Bioscience) – a selective antagonist of MT_2 membrane receptor and N-acetylo-2-benzylotryptamin (Luzindole, Tocris Bioscience) – the non-selective antagonist of MT_1 and MT_2 membrane receptors. Melatonin and 4P-PDOT were dissolved in 95% ethanol and DMSO was used as dissolvent for luzindole.

Cell culture

The murine cancer cell line Colon 38, kindly obtained from Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wroclaw, was used in the experiment. The Colon 38 is transplantable adenocarcinoma originally induced in the colon of C57BL/6 strain mouse by 1,2-dimethylhydrazine (Corbett *et al.* 1975). Adaptation of Colon 38 cells to *in vitro* growth was made by Pajtasz-Piasecka and co-workers (2004).

The continuous culture of the cells was maintained in culture flasks (Nunc Easy flask 25 cm^2, NUNC). The cells were cultured in the present of RPMI 1640 medium (Sigma), supplemented with 25 mM Hepes buffer (Sigma), 100 U/ml penicillin and 100 µg/ml streptomycin solution (Sigma), 4 mM L-glutamine (Sigma), 2 g/l sodium bicarbonate (Sigma) and 5% fetal calf serum (FCS, Biochrom) at 37°C temperature in the humidified atmosphere of 95% air and 5% carbon dioxide. Before confluence (once a week) the cells were harvested after a ten minutes incubation at 37°C in the presence of trypsin-EDTA at the concentrations of 0.05% and 0.02% of Hanks-balanced salt solution (Trypsin-EDTA, Sigma), respectively. Afterwards the cells were washed twice in complete medium and after last centrifugation seeded in a culture flask (2×10^5 cells in 5 ml of a fresh medium) for the four subsequent days.

Experiment

The cells were subjected to the trypsinization process as described above and suspended in the complete medium at a concentration of 4×10^5 cells /ml. Thereafter 50 µl of cell suspension (2×10^4 cells) were placed in the each well of cell culture plates (96 Cell Culture Cluster Dish, Nunclon MicroWell Plates, NUNC) containing 130 µl of complete medium. After 24 hours of preincubation period at 37 °C in the humidified atmosphere of 95% air and 5% carbon dioxide, the 20 µl solution of investigated compounds were added. The cancer cells were cultured for 48 hours in the presence of melatonin at the final concentrations 10^{-7} and 10^{-9} M, 4P-PDOT and luzindole, both at the final concentrations 10^{-6}–10^{-9} M applied alone and melatonin given together with 4P–PDOT or luzindole. The equal volume of culture medium (20 µl) and 95% ethanol (solvent for melatonin and 4P-PDOT) or DMSO (solvent for luzindole) at the highest concentration were added to the appropriate wells with control samples. The cell growth in the culture was measured using EZ4Y system (EZ4Y, Easy for You, The 4th Generation Non Radioactive Cell Proliferation & Cytotoxity Assay, Biomedica Gruppe, Austria, Bellco Biomedica Poland). The assay is based on the transformation of tetrazolium salt into colored soluble formazans as a result of the mitochondrial activity of the viable cells. The red soluble formazans, released to the culture medium, were determined by the extinction measurement using the enzyme-linked immunosorbent assay reader. The optical density (OD) of each sample was measured at 450 nm wave length.

Statistical analysis

The data were statistically analysed by Statgraphics Centurion XV, using a one-way analysis of variance (ANOVA). Statistical differences between tested values were determined using the Least Significant Difference (LSD) test. Data was presented as the means ± SEM. Optical density (OD) in control groups was assumed to be 100 percentage and OD of examined compounds were presented as a percentage of the control group. Differences were considered significant if $p<0.05$.

RESULTS

Melatonin at both examined concentrations 10^{-7} M and 10^{-9} M significantly decreased the viability of cancers cells (Figures 1 and 2). The selective antagonist of MT_2 membrane receptor – 4P-PDOT and luzindole given alone at the concentrations 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M did not influence the growth of Colon 38 cells. The addition of 4P-PDOT to MLT did not change the inhibitory effect of melatonin (Figure 1), whereas luzindole given together with MLT diminished, but failed to block totally, the oncostatic properties of melatonin (Figure 2).

DISCUSSION

The obtained data confirm our earlier observation that melatonin at the physiological concentration (10^{-9} M) and also at the pharmacological level (10^{-7} M) inhibits the growth of murine colonic cancer (Melen-Mucha *et al.* 1998; Winczyk *et al.* 2002; 2001). It is also compat-

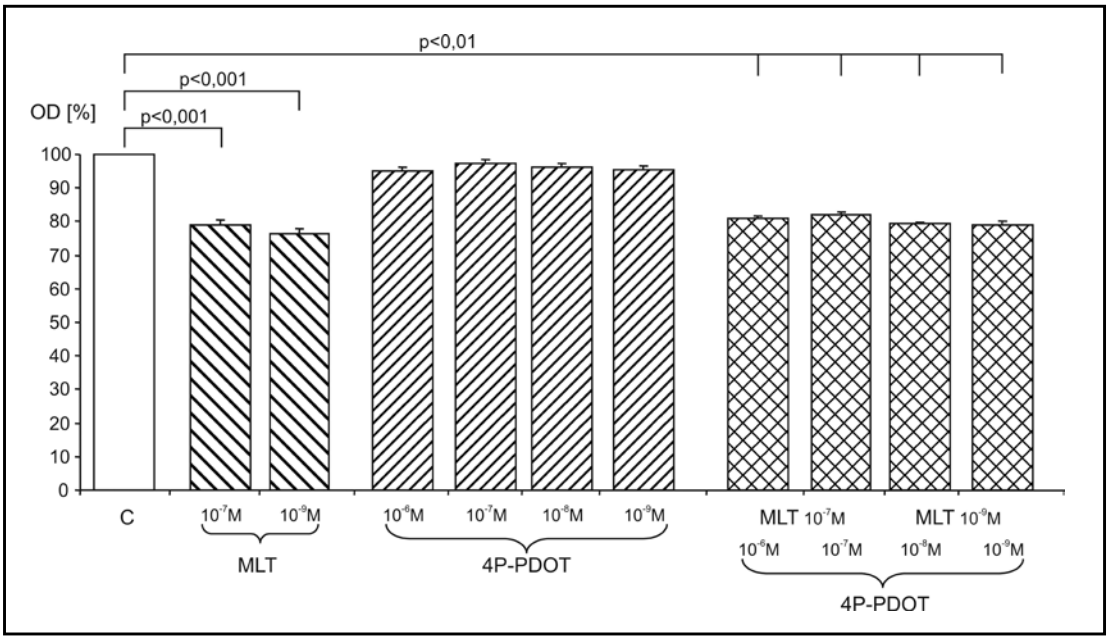

Fig. 1. The effects of melatonin (MLT) and 4-phenylo-2-propionamidotetralin (4-P-PDOT), applied alone and together, on the growth of Colon 38 cancer cells in vitro. Bars represent means ± SEM. C – control

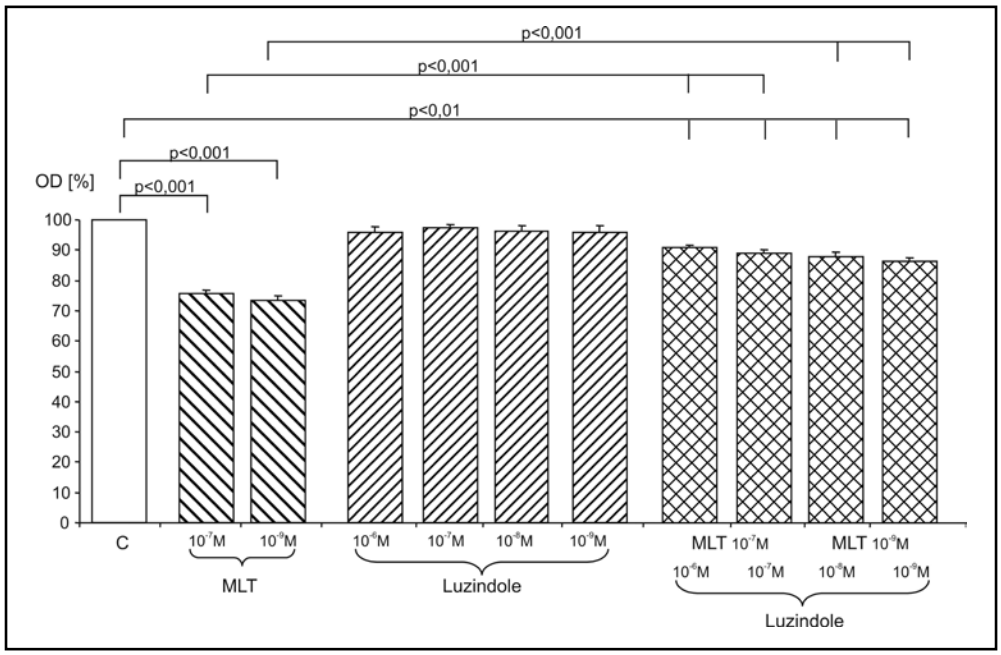

Fig. 2. The effects of melatonin (MLT) and luzindole, applied alone and together, on the growth of Colon 38 cancer cells in vitro. Bars represent means ± SEM. C - control

ible with the findings of Anisimov *et al.* (1997; 2000) showing that MLT inhibits the intestinal carcinogenesis and decreases the growth of carcinogen-induced colon tumors in rats. Melatonin also reduced the proliferation of the cells of murine CT-26 adenocarcinoma and human HT-29 colon cancer but the hormone was applied at high milimolar concentrations (Farriol *et al.* 2000; García-Navarro *et al.* 2007). The specific membrane melatonin receptors have been detected in animal and human intestines (Soták *et al.* 2006; Poon *et al.* 1996; 1997). We have shown that murine Colon 38 cancer possesses both membrane – MT_1 and MT_2 receptors (Karasek *et al.* 2002). The expression of MT_1 receptors and the involvement of this receptor subtype in oncostatic action of MLT have been documented in MCF-7 breast cancer, androgen–dependent LNCaP prostate cancer, PC12 pheochromocytoma and NIE-115 mouse neuroblastoma (Ram *et al.* 2002; Xi *et al.* 2001; Roth *et al.* 2001; Bordt *et al.* 2001). Our earlier study showed that N-[(4-methoxy-1H-indol-2-yl) methyl] propanamide – UCM 386, which is an antagonist of membrane MT_1 receptor and a weak agonist of membrane MT_2 receptor, did not change the inhibitory effect of MLT on Colon 38 cells (Winczyk *et al.* 2002). This observation suggested the participation of MT_2 receptor subtype in oncostatic action of the pineal hormone. The predominant involvement of MT_2 receptors in antiproliferative effects of melatonin has been indicated in ovarian carcinoma, endometrial cancer, choriocarcinoma and melanoma (Shiu *et al.* 1999; Kanishi *et al.* 2000; Petranka *et al.* 1999; Roberts *et al.* 2000. However, the present study shows that the antagonist of membrane MT_2 receptor 4P-PDOT neither has effect alone nor changes the inhibitory action of melatonin on the viability of Colon 38 cells. Similar results have been obtained by other authors (García-Navarro *et al.* 2007). It has been shown that 4P-PDOT does not alter the antiproliferative effect of the pineal hormone on HT-29 human colon cancer cells. Thus, the abovementioned data indicate that the oncostatic action of MLT on human and murine colon cancer is rather independent of subtype MT_2 receptor. Nonetheless, we have observed that luzindole, the antagonist of both membrane melatonin receptors, clearly weakens but fails to block the inhibitory action of MLT on Colon 38 cells. Moreover, we cannot exclude that luzindole may interfere with melatonin action involving some other mechanisms that remain unknown. It is suggested that MLT exerts its anti-cancer effects also via other mechanisms than the modulation of membrane receptors. Melatonin easily enters inside the cells and may act in cytosol via the interaction with calmodulin and also modulates the expression genes by influencing the nucleus directly (Wiesenber *et al.* 1998; Benítez-King *et al.* 1991). In our previous study, the involvement of nuclear RZR/RORα receptors in oncostatic and immunomodulatory effects of melatonin were considered (Wiesenber *et al.* 1998; Garcia-Mauriño *et al.* 1998). The RZR/ROR receptors were cloned in two groups and received the respective names of: retinoid Z receptor (RZR) and retinoid acid receptor-related orphan receptor (ROR) (Becker-André *et al.* 1993; Giguère *et al.* 1994). In human and mammals the α-subtype of these receptor are widely expressed outside the brain, in peripheral tissues. Our data showed that the cells of murine Colon 38 cancer possess the RORα receptors (Karasek *et al.* 2002). Besides, in some

of human cancer cells such as: MCF-7 breast cancer, melanoma, Du-145 androgen-impendent and LNCaP androgen-dependent prostate cancer, RORα transcripts have been also identified (Dai *et al.* 2001; Moretti *et al.* 2000; 2001a; Fischer *et al.* 2006). Although the interaction of melatonin with RZR/RORα receptors is still under discussion, some data supports the participation of these nuclear receptors in oncostatic action of MLT. Melatonin and a thiazolidinedione derivative, CGP 52608, which was characterized as a selective ligand for RZR/RORα receptors, in similar manner inhibit the proliferation of several human cancers cells lines: LNCaP and Du-145 prostate cancers, BG-1 ovarian adenocarcinoma, MCF-7 breast cancer, HT-29 colon cancer (García-Navarro *et al.* 2007; (Dai *et al.* 2001; Moretti *et al.* 2000; 2001a; 2001b; Petranka *et al.* 1999). Moreover, we have found that both compounds exerted comparable anti-proliferative effects on Colon 38 cancer and on rat experimental pituitary tumor (Karasek *et al.* 1998; 1999). Our earlier study also showed that melatonin and CGP 52608 enhance apoptosis in the transplantable murine colon adenocarcinoma (Winczyk *et al.* 2001). The results of the experiments conducted in our laboratory with CGP 55644 – an antagonist of RZR/RORα receptors also support the participation of nuclear receptors in the oncostatic action of the hormone. We have documented that CGP 556444 diminishes the antiproliferative effect of MLT and blocks its proapoptotic action in Colon 38 tumors (Winczyk *et al.* 2002). Moreover, other studies have shown that antagonist of RZR/RORα receptors blocks the inhibitory effect of MLT on the growth of diethylstilbestrol-induced rat prolactin-secreting pituitary tumor cells in vitro (Karasek *et al.* 2003). However, regardless of the outcome reported, the independent effects of both membrane and nuclear receptors of MLT on cancer growth should be considered. It has been shown that the high level of nitric oxide synthase (NOS) correlates with invasiveness and progression of colon carcinoma in humans (Lagares-Garcia *et al.* 2001). Melatonin binding with calmodulin decreases the activity of NOS and in this way may exert its oncostatic action (Pozo *et al.* 1997). The other receptor-independent mechanism in which melatonin may affect cancer growth is its ability to scavenge free radicals and to protect cells from oxidative damage.

Summing up, the present results and our previous study conducted on Colon 38 adenocarcinoma cells indicate that membrane melatonin receptors are not indispensable for the oncostatic action of melatonin and other pathways such as nuclear signaling and receptor-independent mechanism may be also involved.

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