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ORIGINAL ARTICLE

Effects of melatonin and melatonin receptors ligand N-[(4-methoxy-1H-indol-2-yl)methyl]propanamide on murine Colon 38 cancer growth *in vitro* and *in vivo*

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Abstract

OBJECTIVES: Our previous study suggest that oncostatic action of melatonin (MLT) depends mainly on nuclear RZR/ROR receptors. However, we cannot exclude the involvement of membrane receptors in the control of tumor growth. In the present study the effects of MLT and *N*-[(4-methoxy-1H-indol-2-yl)methyl]propanamide (UCM 386 - antagonist of membrane MT_1 receptor and partial agonist of membrane MT_2 receptor) on murine transplantable Colon 38 cancer were investigated *in vitro* and *in vivo* conditions.

MATERIAL AND METHODS: The experiments were performed on adult male B6D2F1 mice strain. *In vitro* the cell proliferation was measured using modified Mosmann method. In the experiment performed *in vivo*, we assessed the cell proliferation, apoptosis and proliferation/apoptosis ratio (P/A). The incorporation of bromodeoxyuridine into tumor cell nuclei was used as an index of cell proliferation (labeling index-LI). The labeling of apoptotic cells according to TUNEL method was considered as an index of apoptosis (AI).

RESULTS: *In vitro* MLT and UCM 386 decreased the cell proliferation, but administration of MLT and UCM 386 together did not change the inhibitory effect of MLT alone. *In vivo* MLT and UCM 386 alone decreased LI and the addition of UCM 386 to MLT did not diminish the antiproliferative effect of MLT. Melatonin and UCM 386 injected alone also increased the AI. Moreover, both compounds given together exerted the additive effect on tumor apoptosis. MLT and UCM 386 alone or together also significantly decreased P/A ratio which is additional parameter confirming the inhibition of tumor growth.

CONCLUSION: The obtained data together with our earlier observations suggest that oncostatic effect of MLT depends on acting via both MT_2 and RZR/ROR nuclear receptors

Introduction

Melatonin (MLT), the hormone of pineal gland, is a new candidate as anticancer agent. Recently, the oncostatic action of melatonin on various experimental cancers has been reported. The antitumor effect of melatonin is connected, in part with antiproliferative and also proapoptotic activities. The number of investigations confirmed the antiproliferative action of melatonin [1, 2, 3]. The previous study conducted in our laboratory on murine Colon 38 cancer shows that MLT causes not only the inhibition of tumor cell proliferation, but also induces apoptosis [4]. The mechanism by which MLT exerts its antitumor effect is very complex and still not clear. This pineal hormone may act indirectly via modulation of endocrine and immune systems. Melatonin may also influence directly on tumor cells through the specific binding sites. The best known melatonin binding sites are two subtypes of receptors, named MT_1 and MT_2 [5]. It was also suggested that MLT is a natural ligand for the nuclear orphan receptors RZR/ROR [6, 7]. Moreover, it was proposed that immunological and oncostatic effects of melatonin depend mainly on nuclear signaling [8, 9]. The involvement of nuclear receptors in the antiproliferative activity of MLT is suggested by the studies in which we have found that MLT and CGP 52608 (selective ligand for RZR/ROR receptors) inhibited the cell proliferation in murine colon cancer and experimental pituitary tumor [10, 11]. Moreover, both the compounds exerted a similar antiproliferative effects on human ovarian adenocarcinoma cell line BG-1 [12] and also on human prostate cancer [13, 14]. It is well documented that MLT acts via high-affinity membrane receptors belonging to the G-protein receptor family [15]. Evidence shown that in mammals MLT acting through membrane receptors regulates mainly circadian and seasonal rhythms [16]. However, it can not be excluded that these membrane receptors are also involved in the control of tumor growth. Therefore, in the present study we investigated whether the MT_1-antagonist and MT_2-partial agonist of membrane receptor *N*-[(4-methoxy-1H-indol-2-yl)methyl]propanamide given together with melatonin changes the antitumor effects of melatonin.

Materials and methods

Compounds

The following substances were examined in this study: melatonin (N-acetyl-5-methoxytryptamine; Sigma) and *N*-[(4-methoxy-1H-indol-2-yl)methyl]propanamide (UCM 386). UCM 386, synthesized in the Institute of Pharmaceutical Chemistry at the University of Urbino, is antagonist of MT_1 receptor (pKi ± SEM = 6.39±0.06, the relative intrinsic activity IA_r=0.05) and partial agonist of MT_2 receptor (pKi ± SEM = 6.54 ±0.02, the relative intrinsic activity IA_r=0.3) [17]. The structures of both compounds are shown in Fig. 1.

Animals

Ten weeks old male B6D2F1 mice, weighing about 30±4g were used in this experiment. B6D2F1 strain is the first generation of the cross-bred between C57BL/6 and DBA/2 strains of mice. The animals were kept under 12-hr/12-hr light/dark cycle (lights on from 08:00 to 20:00h) and the temperature was maintained at 22±2°C, with access to food and tap water *ad libitum*. The experiments were performed in February.

Tumor induction

The Colon 38 is transplantable adenocarcinoma originally induced in the colon of C57BL/6 mice by 1,2-dimethylhydrazine [18]. In the present study the induction of tumor was conducted by a subcutaneous injection of 0.2 ml of a 33% suspension of Colon 38 cancer cells into axillary region.

In vitro study

Four weeks after injections of cancer cells animals were killed by spinal cord dislocation. All detectable tumors were aseptically removed and fixed in complete culture medium (RPMI-1640, Sigma) supplemented with gentamicin (KRKA) (5 μg/100ml) and 10% heat inactivated fetal calf serum (FCS, Biochrom K.G.). After tumors had been gently rinsed with complete culture medium three times they were chopped with a sharp, thin scissors into small pieces of 0.5 mm length and quickly mechanically filtered through a metal strainer. Then the cell suspension was filtered again through a fine mesh nylon (Cell strainer nylon, 70 μm, Falcon) and the cells were resuspended in fresh complete culture medium to obtain the suspension of $3x10^6$ cells/ml. 100 μl of the cell suspension ($3x10^5$ cells) were placed in the wells of cell culture plates (96 Cell Culture Cluster Dish, Costar; Nunclon, Microwell Plates, NUNC) and incubated for 1 hr (5%CO_2, 37° C, 95% humidity). Then both tested substances were added to the appropriate wells:

- melatonin at the final concentrations 10^{-7} M,
- UCM 386 at the final concentrations 10^{-7} M,
- UCM 386 (10^{-7} M) + melatonin (10^{-7} M).

The melatonin concentration of 10^{-7} M was used on basis of our preliminary unpublished studies in which we tested the influence of different melatonin concentrations on tumor growth.

The equal volume of culture medium was added to the control wells. The final volume of culture medium

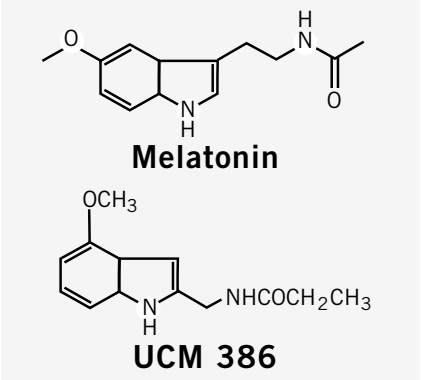


Fig. 1. The structure of melatonin and UCM 386.

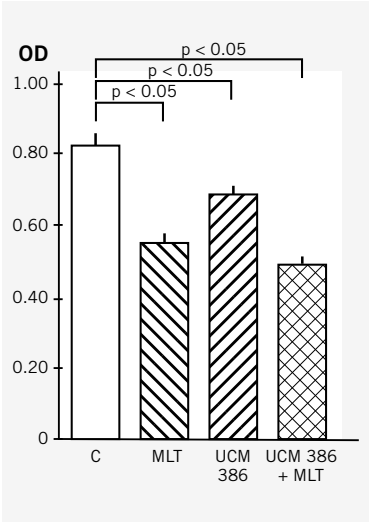


Fig. 2. Effects of melatonin (MLT) and UCM 386 on optical density of sample (OD) in Colon 38 cancer cells *in vitro*. Bars represent means±SEM.

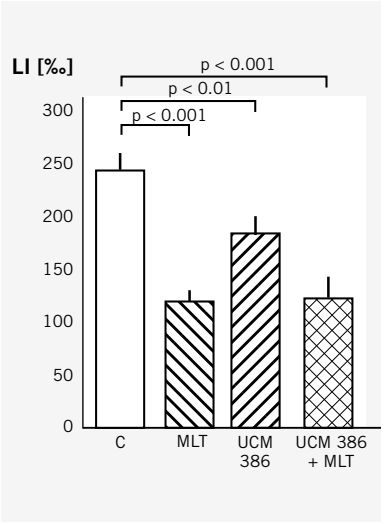


Fig. 3. Effects of melatonin (MLT) and UCM 386 on bromodeoxyuridine labeling index (LI) in Colon 38 cancer cells *in vivo*. Bars represent means±SEM.

and substances in each well was 200 μl. After 48 hrs of incubation (as described above) the cell proliferation was measured using modified Mosmann method (EZ4Y, Easy for You, The 4th Generation Non Radioactive Cell Proliferation & Cytotoxity Assay, Biomedica Gruppe, Austria, Bellco Biomedica Poland). The optical density of each sample (OD) was measured by microplate reader at 450 nm.

In vivo study

Ten days after induction tumor the animals received the examined substances as follows: group I (control) – 0.25 ml physiological saline; group II – melatonin-25 μg per animal s.c.; group III – UCM 386 – 25 μg per animal s.c.; group IV – UCM 386 + melatonin at the above doses per animal s.c.. The substances were given once daily in the evening at 18:00 h for six days. Fourteen hours after the last injection all animals received a single intraperitoneal injection of bromodeoxyuridine (BrdU, Sigma) at a dose of 50 mg/kg b.w. Ninety minutes later the mice were killed by spinal dislocation. All tumors were removed and fixed in Bouin's fluid. The tissues were embedded in paraffin.

Three parameters have been measured in this study: the cell proliferation, the apoptosis and the proliferation/apoptosis ratio.

The bromodeoxyuridine labeling index (LI) as an index of cell proliferation was detected by the immunocytochemical method. The BrdU is a pyridine analogue capable of being incorporated into DNA in place of thymidine during S phase of the cell cycle. We used the mouse immunoglobulins (Monoclonal Mouse Anti- Bromodeoxyuridine, Dako) as a primary antibodies. Next we employed Strep ABC complex Duet Reagent Set, Dako which consisting of the biotynylated goat antibody and the streptavidin complexed with biotynylated peroxidase. The number of BrdU immunopositive nuclei per 1000 randomly scored tumor cells were counted in the microscopic preparation.

The apoptotic cells were identified using the terminal deoxynucleotidyl transferase-mediated dUTPnick end labelling (TUNEL) method. The sections were incubated for 15 min. at room temperature in 20 μg/ml of proteinase K (Sigma). Endogenous proteinase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol for 30 min. Subsequently, the terminal transferase reaction was performed using In Situ Cell Detection Kit (POD Boehringer, Mannheim). Unspecific antibody binding was blocked by preincubation with 5% normal sheep serum. Detection of immunoreactivity was performed using 3'3'-diaminobenzidine as a chromogen. Finally, the sections were counterstained with haematoxylin. A negative control was obtained by omitting the incubation with terminal deoxynucleotidyl transferase. Under 1000x magnification the number of cells containing apoptotic bodies or nuclei was counted in 1000 randomly chosen tumor cells.

Statistical analysis

The results from both experiments are expressed as the means ± SEM. Statistical comparisons between experimental groups were made with nonparametric Mann-Whitney's test.

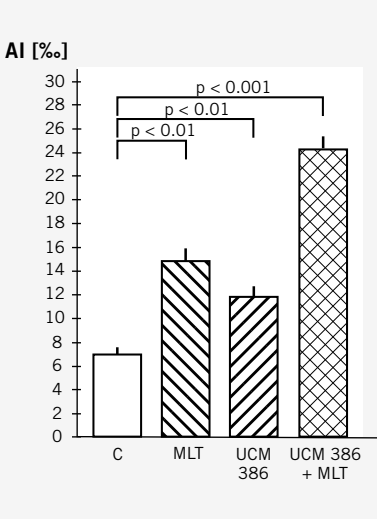
Results

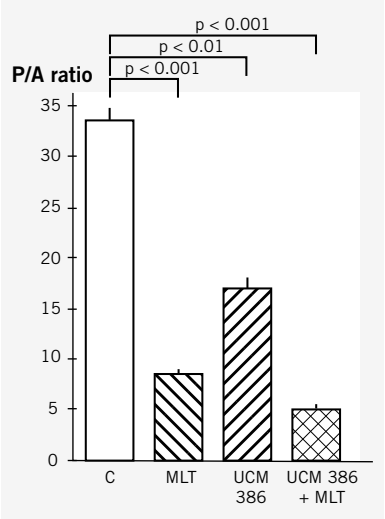
In the experiment *in vitro* melatonin decreased strongly the number of living cells. UCM 386 also inhibited the proliferation of colon cancer cells but not so strongly as melatonin. Moreover, the administration of MLT and UCM 386 jointly did not change the inhibitory effect of MLT alone (Fig. 2).

In the experiment *in vivo*, melatonin inhibited the cell proliferation of murine colon cancer. UCM 386 also decreased the LI, but its action was less effective than action of melatonin. Moreover the addition of UCM 386 to melatonin did not diminish the antiproliferative action of MLT (Fig. 3). In the present study melatonin similarly to our earlier results enhanced the number of apoptotic cells. Antagonist of MT_1 membrane receptor given alone also increased the apoptotic index. Unexpectedly, the MLT and UCM 386 given together exerted the additive effect on tumor apoptosis and caused very strong increase in the number of apoptotic cells as compared to control group (Fig. 4). Proliferation/apoptosis ratio is an additional parameter assessing the tumor growth. The

Fig. 4. Effects of melatonin (MLT) and UCM 386 on apoptotic index (AI) in Colon 38 cancer cells *in vivo*. Bars represent means±SEM.

Fig. 5. Effects of melatonin (MLT) and UCM 386 on proliferation/apoptosis ratio (P/A) in Colon 38 cancer cells *in vivo*. Bars represent means±SEM.





low P/A ratio may indicate that tumor growth is inhibited. Compared to the control group MLT alone or given together with UCM 386 very strongly lowered P/A ratio (Fig. 5). The UCM 386 given alone was less effective, but its action was also significant.

Discussion

The data presented above confirm our earlier observation that the direct oncostatic action of melatonin depends on both antiproliferative and proapoptotic effects. The antiproliferative activity of pineal hormone was revealed in studies performed on the hormone-dependent cancers (human breast cancer [19], endometrial cancer [20], ovarian adenocarcinoma [12]) and also on tumors, which are not hormone-dependent [21, 22]. It is worth to underline that the changes revealed *in vitro* using the EZ4U method correspond well with the changes of P/A ratio obtained *in vivo*. It seems reasonable since both parameters reflect the growth potential, which is a balance between cell proliferation, and cell death. Melatonin as a natural antioxidant and free radical scavenger should exhibit rather antiapoptotic action [23]. Indeed, the prevention of apoptosis by this hormone has been observed in the experiments concerning mainly non-tumor cells [24, 25]. However, our previous study and present experiment showed that melatonin enhanced apoptosis of murine colon cancer. Our results corroborate with the observation of Eck et al. [26], which proved that melatonin and retinoic acid induced apoptosis in MCF-7 mammary cancer cells. Anisimov et al. [27, 28] conducted the study on intestinal tumor similar to Colon 38 adenocarcinoma. They have found that melatonin inhibited the development, cell proliferation, multiplicity, and also enhanced the number of apoptotic cells in 1,2-dimethylhydrazine-induced intestinal tumors. The mechanism of oncostatic action of melatonin has been not completely understood so far. Our earlier observations, confirmed by others, showing that melatonin and the selective ligand of RZR/ROR receptor have similar antiproliferative effect on cancer cells might suggest the involvement of nuclear signaling. However, the results of present study may also confirm the participation of the membrane receptors in control of tumor growth. Inhibition of cell proliferation by UCM 386, which is an antagonist of MT_1 receptor and weak agonist of MT_2 receptor, may establish the involvement of MT_2 subtype receptor. These findings are compatible with observation of Kinishi et al. [20] showing that antiproliferative action of MLT on Ishikawa endometrial cancer cells was blocked by pre-treatment with luzindol, a selective antagonist of MT_2 receptor. Besides, we have found that UCM 386 injected alone significantly increased AI of Colon 38 tumor cells. Moreover UCM 386 and MLT given together caused additive effect on tumor apoptosis. The explanation of these results is difficult. Recently, it has been suggested that MLT can change the phosphorylation state of RZR/ROR receptors via membrane receptor and in this way involves apoptosis [9]. We may speculate that UCM 386 induces apoptosis altering the phosphorylation state of RZR/ROR receptors only via MT_2 receptor. On the other hand, MLT may influence the nuclear receptor not only via MT_2 receptor, but also interacts directly with RZR/ROR receptors. However, the experiments using the nuclear RZR/ROR and membrane MT_2 receptor antagonists are needed to confirm or reject this hypothesis.

In summary, our results suggest that direct oncostatic effect of melatonin depends on acting via both MT_2 membrane and RZR/ROR nuclear receptors. This hypothesis is supported by demonstration of MT_2 and RORα_1 receptors' expression in Colon 38 cells (29). Further studies are needed to elucidate the complex pathways of antitumor melatonin action.

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REFERENCES

1 Panzer A , Viljoen M. The validity of melatonin as an oncostatic agent. J Pineal Res 1997; **22**:184–202.

2 Karasek M, Pawlikowski M. Pineal gland, melatonin and cancer. Neuroendocrinol Lett 1999; **20**: 139–44.

3 Pawlikowski M, Winczyk K, Karasek M. Oncostatic action of melatonin: facts and question marks. Neuroendocrinol Lett 2002; **23**(suppl 1):24–29.

4 Melen-Mucha G, Winczyk K, Pawlikowski M. Somatostatin analogue octreotide and melatonin inhibit bromodeoxyuridine incorporation into cell nuclei and enhance apoptosis the transplantable murine Colon 38 cancer. Anticancer Res 1998; **18**:3615–20.

5 Dubocovich ML, Cardinali DP, Delagrange P, Krause DN, Strosberg D, Sugden D, et al. Melatonin receptors. In: The IUPHAR Compendium of receptor characterization and classification. London: IUPHAR Media, UK. 2001, p. 270–7.

6 Calberg C, Wiesenberg I. The orphan receptor family RZR/ROR, melatonin and 5-lipooxygenase: An unexpected relationship. J Pineal Res 1995; **18**:171–8.

7 Steinhilberg D, Carlberg C. Melatonin receptor ligands. Exp Opin Ther Patents 1999; **9**:281–90.

8 Garcia-Maurino S, Gonzales-Haba MG, Calvo JR, Goberna R, Guerrero JM. Involvement of nuclear binding sites for melatonin in the regulation of IL-2 and IL-6 production by human blood mononuclear cells. J Neuroimmunol 1998; **92**:76–84.

9 Wiesenber I, Missbach M, Carlberg C. The potential role of the transcription factor RZR/ROR as a mediator of nuclear melatonin signalling. Restr Neurol Neurosci 1998; **12**:143–50.

10 Karasek M, Winczyk K, Kunert-Radek J, Wiesenberg I, Pawlikowski M. Antiproliferative effects of melatonin and CGP 52608 on murine Colon 38 adenocarcinoma in vitro and in vivo. Neuroendocrinol Lett 1998; **19**:71–8.

11 Pawlikowski M, Kunert-Radek J, Winczyk K, Melen-Mucha G, Gruszka A, Karasek M. The antiproliferative effects of melatonin on experimental pituitary and colonic tumors. Adv Exp Med Biol 1999; **460**:369–72.

12 Petranka J, Baldwin W, Biermann J, Jayadev S, Barrett JC, Murphy E. The oncostatic action of melatonin in an ovarian carcinoma cell line. J Pineal Res 1999; **26**:129–36.

13 Marelli M, Limonta P, Maggi R, Motta M, Moretti R.M. Growth-inhibitory activity of melatonin on human androgen-independent DU 145 prostate cancer cells. Prostate 2000; **45**:238–44.

14 Moretti RM, Marelli MM, Motta M, Polizzi D, Monestiroli S, Pratesi G, Limonta P. Activation of the orphan nuclear receptor ROR alpha induces growth arrest in androgen-independent DU 145 prostate cancer cells. Prostate 2001; **46**:327–35.

15 Morgan PJ, Barret P, Howell HE, Helliwell R. Melatonin receptors: Localization, molecular pharmacology and physiological significance. 1994; **24**:101–46.

16 Dubocovich ML. Melatonin receptors: are there multiple subtypes? Trends Pharmacol Sci 1995; **16**:50–6.

17 Spadoni G, Balsamini C, Diamantini G, Tontini A, Tarzia G, Mor M, et al. 2-*N*-acylaminoalkylindoles: design and quantitative structure-activity relationship studies leading to MT_2-selective melatonin antagonists. J Med Chem 2000; **44**:2900–12.

18 Corbett TH, Griswold DP, Jr, Roberts BJ, Peckham C, Schabel FM, Jr. Tumor induction relationship in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. Cancer Res 1975; **35**:2434–39.

19 Ram PT, Yuan L, Dai J, Kiefer T, Klotz DM, Spriggs LL, et al. Differential responsiveness of MCF-7 human breast cancer cell line stocks to the pineal hormone, melatonin. J Pineal Res 2000; **28**:210–8.

20 Kanishi Y, Kobayashi Y, Noda S, Ishizuka B, Saito K. Differential growth inhibitory effect of melatonin on two endometrial cancer cell lines. J Pineal Res 2000; **28**:227–33.

21 Cos S, Verduga C, Femandez-Viadero C, Megias M, Crespo D. Effects of melatonin on the proliferation and differentiation of human neuroblastoma cells in culture. Neurosci Lett 1996; **216**:113–6.

22 Farriol M, Venereo Y, Orta X , Castellanos JM, Segovia-Silvestre T. In vitro effects of melatonin on cell proliferation in colon adenocarcinoma line. J Appl Toxic 2000; **20**:21–4.

23 Reiter RJ, Tan DX, Cabrera J, D'Arpa D, Sainz RM, Mayo JC, et al. The oxidant/antioxidant network: Role of melatonin. Biol Signals Recept 1999; **8**:56–63.

24 Cagnoli CM, Atabay C, Kharlamova E, Manev H. Melatonin protects neurons from singlet oxygen-induced apoptosis. J Pineal Res 1995; **18**:222–6.

25 Provinciali M, Di Stefano G, Bulian D, Tibaldi A, Fabris N. Effect of melatonin and pineal grafting on thymocytes apoptosis in aging mice. Mech Age Develop 1996; **90**:1–19.

26 Eck KM, Yan L, Duffy L, Ram PT, Ayetty S, Chen I, et al. A sequential treatment regimen with melatonin and all-trans retinoic acid induces apoptosis in MCF-7 tumour cells. Br J Cancer 1998; **77**:2129–37.

27 Anisimov VN, Popovich IG, Zabezhinski MA. Melatonin and colon carcinogenesis: I. Inhibitory effect of melatonin on development of intestinal tumors induced by 1,2-dimethylhydrazine in rats. Carcinogenesis 1997; **18**:1549–53.

28 Anisimov VN, Popovich IG, Shtylik AV, Zabezhinski MA, Ben-Huh H, Gurevich P, et al. Melatonin and colon carcinogenesis: Effect of melatonin on proliferative activity and apoptosis in colon mucosa and colon tumors induced by 1,2-dimethylhydrazine in rats. Exp Toxicol Pathol 2000; **52**:71–6.

29 Karasek M, Carrillo-Vico A, Guerrero JM, Winczyk K, Pawlikowski M. Expression of melatonin MT_1 and MT_2 receptors, and RORα_1 receptor in transplantable murine Colon 38 cancer. Neuroendocrinol Lett 2002; **23**(suppl 1):55–60.