# Growth-inhibitory action of melatonin and thiazolidinedione derivative CGP 52608 on murine 16/C breast cancer cells

**Katarzyna Winczyk [1], Hanna Lawnicka [2], Marek Pawlikowski [1], Jolanta Kunert-Radek [3] & Michal Karasek [1,4]**

[1] Department of Neuroendocrinology, Chair of Endocrinology, Medical University of Lodz, Poland.
[2] Department of Immunoendocrinology, Chair of Endocrinology, Medical University of Lodz, Poland.
[3] Clinic of Endocrinology, Chair of Endocrinology, Medical University of Lodz, Poland.
[4] Department of Endocrinology and Metabolic Diseases, Polish Mother's Memorial Hospital – Research Institute, Lodz, Poland.

*Correspondence to:* Prof. Dr. Michal Karasek
Department of Neuroendocrinology,
Chair of Endocrinology, Medical University of Lodz
92-216 Lodz, Czechoslowacka 8/10, POLAND
PHONE/FAX: +48 42 675 76 13
EMAIL: karasek@csk.umed.lodz.pl

*Submitted: February 22, 2006* *Accepted: February 26, 2006*

*Key words:* **melatonin; CGP 52608; RZR/ROR receptors; cell proliferation; breast cancer**

Neuroendocrinol Lett 2006; **27**(3):351–354 **PMID:** 16816834 NEL270306A12  **www.nel.edu**

## Abstract

**OBJECTIVES:** Melatonin may influence directly tumor cells through the specific binding sites. The best known melatonin binding sites are membrane receptors. Recently, the participation of nuclear signalling via estrogen as well as RZR/ROR receptors in oncostatic action of melatonin on the breast cancer has been widely discussed. The aim of present study was to investigate effects of melatonin, the selective ligand for nuclear RZR/ROR receptors – CGP 52608, and methotrexate on growth of murine 16/C breast cancer cells.

**MATERIAL AND METHODS:** The experiment was performed in vitro. The breast cancer cells were incubated for 2 days in the presence of melatonin, CGP 52608 (at concentrations of $10^{-5}$M, $10^{-7}$M, $10^{-9}$M, $10^{-11}$M ) and methotrexate (at concentrations of 0.25 and 0.125 μg/ml). The growth of cells was measured using the modified Mossman method.

**RESULTS:** All examined compounds significantly inhibited the growth of cancer cells. The effects of MLT and CGP 52 608 were comparable with suppression caused by methotrexate. The significant differences of efficacy between two examined concentrations of methotrexate were not observed.

**CONCLUSION:** The obtained data together with our previous results indicate that nuclear receptors RZR/ROR play an important, although not sufficiently recognized role in the oncostatic action of melatonin.

## Introduction

Different therapeutic models are applied in the treatment of breast cancer, but their effectiveness is still not satisfying. Therefore, the new drugs increasing anticancer effect for this pathology are investigated. Melatonin (MLT), the main hormone of pineal gland is a new candidate as oncostatic agent. Several of experimental study conducted on animal and human cell lines, and also on experimentally induced animal tumors have confirmed the oncostatic properties of pineal hormone [22].



ORIGINAL ARTICLE

The mechanism by which MLT inhibits tumor growth is very complex. Among different ways such as the modulation of endocrine [27] and immune system [16], and the antioxidative action [14]. MLT influences target cells directly via the specific receptors [12]. At present, it is believed that MLT may act not only through $MT_1$ and $MT_2$ membrane receptors [6], but also via nuclear RZR/ROR receptors [29]. The involvement of nuclear receptor in oncostatic action of MLT is suggested by our and other studies assessing effect of CGP 52608 (selective ligand for nuclear RZR/ROR receptors) [11, 21, 23]. We have found that MLT and thiazolidinedione CGP 52608 inhibited, in similar manner, the cell growth of murine colonic cancer [11] and diethylstilbestrol-induced rat pituitary tumor [21]. Additionally, the other authors have shown the similar antiproliferative effects of both compounds on human ovarian adenocarcinoma cell line BG-1 [23] and on human androgen-dependent (LNCaP) [18, 19] as well as on androgen-independent (DU 145) prostate cancers [17, 20]. There is an evidence, coming mainly from the experimental studies, indicating the oncostatic action of MLT on breast cancer [3, 7, 9], but data on CGP 52608 effect are rather rare [4]. Thus, we decided to examine the influence of both agents on murine adenocarcinoma cell line 16/C and to compare their effects with action of methotrexate, one of the drugs applied in the therapy of breast cancer.

## Material and methods

*Cell culture*

The murine adenonocarcinoma cell line 16/C, kindly obtained from Hirszfeld Institute of Immunology and Experimental Therapy, Wroclaw, was used in the experiment. The continuous culture of the cells was maintained in culture flasks (Nunc Easy flask 25 $cm^2$, NUNC). The cells were cultured in RPMI 1640 medium (Sigma) supplemented with 25 mM Hepes buffer (Sigma), 100 U/mL penicillin and 100 μg/ml streptomycin solution (Sigma) and 10% heat-inactivated bovine fetal serum (FBS, Biochrom) at 37°C in the humidified atmosphere of 95% air and 5% $CO_2$. Before confluence (twice a week) the cells were harvested after a 10-min incubation at 37°C the presence of trypsin-EDTA (0.05 and 0.02% respectively) in Hanks-balanced salt solution (Sigma). The cells were washed three times in complete RPMI and after last centrifugation seeded at $10^6$ cells in 5 mL of fresh medium.

*Experiment*

The cells were subjected to the trypsynization process as described above and suspended at $1x10^6$/mL cells in complete RPMI. $5x10^4$ cells (50 μl of cell suspension) were placed in the wells of cell culture plates (96 Cell Culture Cluster Dish, Nunclon MicroWell Plates, NUNC) containing 130 μL of complete RPMI. After 24 hrs of incubation (5%$CO_2$, $37^0$C, 95% humidity), the 20 μL of investigated compounds: methotrexate (Ebeve) in final concentrations 0.25 μg/mL and 0.125 μg/mL, melatonin (Sigma, USA) and CGP 52608 (Novartis Pharma Inc., Basel, Switzerland) in final concentrations $10^{-5}$M, $10^{-7}$M, $10^{-9}$M, $10^{-11}$M were added to the appropriate wells. Melatonin and CGP 52608 were both dissolved firstly in 0.9% NaCl with 10% of 95% ethanol and then in RPMI-1640. The highest concentration of 95% ethanol in wells was 0.13% (in samples with $10^{-5}$M of melatonin and CGP 52608). The equal volume of culture medium (20 μL) and 95% ethanol (in final concentration 0.13%) was added to the control samples. After 48hrs of incubation the cell proliferation 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.

*Statistical analysis*

The data was presented as the means ± SEM. Statistical comparisons between experimental groups were determined with nonparametric Mann-Whitney's test. Differences were considered significant if $p<0.05$.

## Results

All examined compounds significantly inhibited the growth of murine 16C breast cancer cells. As can be seen in Fig. 1, melatonin was the most effective at concentration of $10^{-7}$M. Action of CGP was the strongest at concentration of $10^{-5}$M. The effects of MLT and CGP were comparable with suppression caused by methotrexate. No significant differences between two examined concentrations of methotrexate were observed.

## Discussion

On the basis of the previous reports, it can be stated that a direct action of melatonin on breast cancer may depend on the membrane receptors or/and the nuclear RZR/ROR receptors and also depends on the presence of estrogen receptors (ER) in tumor cells. Immunohistochemical examination showed $MT_1$ membrane receptors in normal and malignant human breast tissues, however, in tumor cells high receptors levels occurred [5]. Expression of $MT_1$ but not $MT_2$ receptors has been also found in MCF-7 human breast cancer [25]. Furthermore, overexpression of the $MT_1$ receptor in MCF-7 cancer cells reduces tumor incidence in mice receiving MLT [1] and enhances the antiproliferative effect of MLT on breast cancer cells [32]. Ram et al. [25] have shown that pineal hormone inhibits the cell proliferation by activation of $MT_1$ receptor and melatonin's growth-inhibitory effect occurs only in breast cancer cells having ER. The relationship between the expression of ER and the antitumor action of MLT has been the object of extensive investiga-

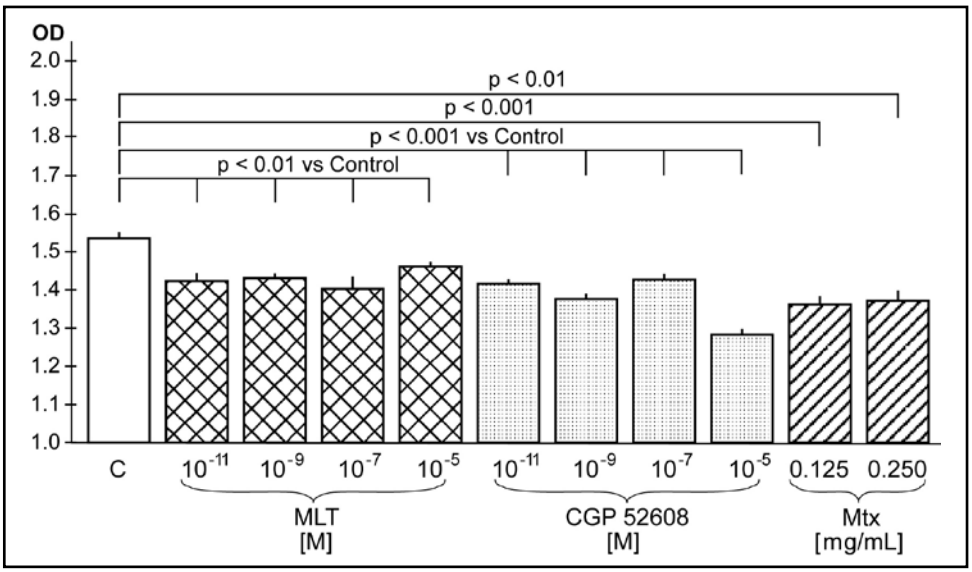


**Fig. 1.** Effects of melatonin (MLT), the ligand of RZR/ROR receptor – CGP 52608 and methotrexate (Mtx) on optical density of sample in 16/C cancer cells in vitro; C-control group. Bars represent means ± SEM.

tions in recent years [28]. It has been proved that there is correlation between level of ER in tumor cells and the oncostatic effect of MLT on breast and other types of cancers [10, 24]. It was suggested that MLT acts through destabilization of binding of the estradiol-estrogen receptor complex to the estrogen responsive element on DNA [26]. In the present study we observed the inhibitory-growth effect of MLT on 16/C breast cancer cells. 16/C mouse mammary adenocarcinoma was derived from lung metastasis of mammary cancer spontaneously arisen in female C3H mouse [2] and was used for anticancer drug screening [15]. It has been known that these cancer cells possess ER, but the expressions of membrane melatonin and nuclear RZR/ROR receptors have been not examined. However, the involvement of the estrogen receptors can not be excluded. Similar inhibitory effect of CGP 52608 and MLT observed in our experiment suggests the participation of nuclear signalling in the oncostatic action of MLT. The contribution of RZR/ROR receptors in action of MLT has been lately investigated by our and other groups [4, 11, 21, 23]. We have found that MLT and RZR/RORα receptor ligand CGP 52608 exerted similar antiproliferative effects on colonic cancer cells having nuclear RORα receptors [11, 13]. Moreover, the study conducted in our laboratory on murine Colon 38 cancer has proved that MLT and CGP as well, cause not only the inhibition of tumor proliferation, but also induce apoptosis [30]. In the next our investigations we showed that thiazolidinedione CGP 55644 (an antagonist of nuclear RZR/ROR receptor) blocked the proapoptotic effect of pineal hormone [31]. These results suggest that the induction of apoptosis by MLT depends mainly on its action via nuclear RZR/RORα receptors. Transcripts of RORα receptors have been identified in the ER-positive MCF-7 cancer cells as well as in the breast cancers, which do not possess ER [4, 8, 25]. It was found also that melatonin inhibits the transcriptional activity of RZR/ROR receptors in MCF-7 breast cancer [4]. Besides, both MLT and CGP 52608, RZR/RORα agonist, at a concentration of $10^{-9}$M, similarly decrease MCF-7 cell proliferation in time-dependent manner [25]. However, the data concerning the effects of MLT in dependence of RZR/ROR expression are controversial. Ram et al. [25] have observed, that MLT, and also ligand RZR/ROR did not inhibit the growth of breast cancer cells, which are ER-negative and express only trace amount $MT_1$. On the other hand, Girgert et al. [8] have shown that in the breast cancers cells possessing higher expression of RZR/ROR but not expressing $MT_1$ receptors, MLT was most effective at lower concentrations.

In summary, results of the present study showed that melatonin and the selective ligand of RZR/ROR receptors have similar antiproliferative effect on 16/C breast adenocarcinoma cells and action of both compounds are comparable with suppression caused by methotrexate. Above data, together with earlier findings coming from our and other laboratories indicate that nuclear receptors RZR/ROR play an important, although not sufficiently recognized role in the oncostatic action of melatonin.

REFERENCES

1 Collins A, Yuan L, Kiefer TL, Cheng Q, Lai VL, Hill SM. Overexpression of the MT1 melatonin receptor in MCF-7 human breast cancer cells inhibits mammary tumor formation in nude mice. Cancer Lett 2003; **189:**49–57.
2 Corbett TH, Grisworld DP, Roberts BJ, Peckham JC, Schabel FM. Biology and therapeutic response of mouse mammary adenocarcinoma (16/C) and its potential as model for surgical adjuvant chemoterapy. Cancer Treat Rep 1978; **10:**1471–88.
3 Cos S, Sanchez-Barcelo EJ. Melatonin inhibition of MCF-7 human breast-cancer cells growth: influence of cell proliferation rate. Cancer Lett 1995; **93:**207–12.
4 Dai J, Ram PT, Yuan L, Spriggs LL, Hill SM. Transcriptional repression of RORalpha activity in human breast cancer cells by melatonin. Mol Cell Endocrinol 2001; **176:**112–20.

5 Dillon DC, Easley SE, Asch BB, Cheney RT, Brydon L, Jockers R. Differential expression of high-affinity melatonin receptors (MT1) in normal and malignant human breast tissue. Am J Clin Pathol 2002; **118:**451–8.
6 Dubocovich ML, Cardinali DP, Delangrange P, Krause DN, Strosberg D, Sugden D, et al. Melatonin receptors. In: The IUPHAR compendium of receptor characterization and classification.2nd ed. London: IUPHAR Media, UK. 2001, pp. 270–7.
7 Eck KM, Yuan L, Duffy L, Ram PT, Ayettey S, Chen I. A sequential treatment regiment with melatonin and all-trans retinoic acid induces apoptosis in MCF-7 tumor cells.Br J Cancer 1998; **77:**2129–37.
8 Girgert R, Bartsch C, Hill SM, Kreienberg R, Hanf V. Tracking the elusive antiestrogenic effect of melatonin: A new methodological approach. Neuroendocrinology Lett 2003; **24:**440–4
9 Hill SM, Blask DE. Effects of the pineal hormone melatonin on the proliferation and morphological characteristics of human cancer cells (MCF-7) in culture. Cancer Res 1988; **48:**6121–6.
10 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.
11 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.
12 Karasek M, Pawlikowski M. Pineal gland, melatonin and cancer. Neuroendocrinoll Lett 1999; **20:**184–202.
13 Karasek M, Carrillo-Vico A, Guerrero JM, Winczyk K, Pawlikowski M. Expression of melatonin $MT_1$, $MT_2$ receptors and $ROR\alpha_1$ receptor in transplantable murine Colon 38 cancer. Neuroendocrinology Lett 2002; **23 (suppl.1)**:55–60.
14 Karbownik M, Reiter RJ, Burkhardt S, Gitto E, Tan DX, Lewinski A. Melatonin attenuates estradiol-induced oxidative damage to DNA: relevance for cancer prevention. Exp Biol Med 2001; **226:**707– 12.
15 Kusmierczyk H, Radzikowski C, Paprocka M, Budzyński W, Rak J, Kinas R. Antitumor activity of optical isomers of cyclophosphamide, ifosfamide and trofosfamide as compared to clinically used racemates. J Immunopharmacology 1986; **4:**455–80.
16 Maestroni GJM. The immunoneuroendocrine role of melatonin. J Pineal Res 1993; **14:**1–10.
17 Montagnani Marelli M, Limonta P, Maggi R, Motta M, Moretti RM. Growth-inhibitory activity of melatonin on human androgen-independent DU 145 prostate cancer cells. Prostate 2000; **45:**238–44.
18. Moretti RM, Montagnani Marelli M, Maggi R, Dondi D, Motta M, Limonta P. Antiproliferative action of melatonin on human prostate cancer LNCaP. Oncol Rep 2000; **7:**347–51.
19 Moretti RM, Montagnanii Marelli M, Motta M, Limonta P. Oncostatic activity of a thiazoldinedione derivative on human androgen-dependent prostate cancer cells. Int J Cancer 2001; **92:** 733–7.
20 Moretti RM, Montagnanii Marelli M, Motta M, Pollizzi D, Monestiroli S, Pratesi G, et al. Activation of the orphan nuclear receptor ROR alpha induces growth arrest in androgen-independent DU 145 prostate cancer cells. Prostate 2001; **46:**327–35.
21 Pawlikowski M, Kunert-Radek J, Winczyk K, Meleń-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.
22 Pawlikowski M, Winczyk K, Karasek M. Oncostatic action of melatonin: facts and question marks. Neuroendocrinol Lett 2002; **23 (suppl 1):**24–9.
23 Petranka J, Baldwin W, Biermann J, Jayadev S, Barret JC, Murphy E. The oncostatic action of melatonin in an ovarian carcinoma cell line. J Pineal Res 1999; **26:**129–36.
24 Ram PT, Yuan L, Dai J, Kiefer TL, Klotz DM, Spriggs. Differential responsiveness of MCF-7 human breast cancer cell line stocks to the pineal hormone, melatonin. J Pineal Res 2000; **28:**210–8.
25 Ram PT, Dai J, Yuan L, Dong C, Kiefer TL, Lai VL, et al. Involvement of mt1 melatonin receptor in human breast cancer. Cancer Lett 2002; **179:**141–50.
26 Rato AG, Pedrero JG, Martinez MA, del Rio B, Lazo PS, Ramos S. Melatonin blocks the activation of estrogen receptor for DNA binding. FESEB J 1999; **13:**857–68.
27 Reiter RJ. The pineal and its hormone on the control of reproduction in mammals. Endocr Rev 1980; **1:**109–31.
28 Sanchez-Barcelo EJ, Cos S, Mediavilla D, Martinez-Campa C, Gonzales A, Alonso-Gonzales C. Melatonin-estrogen interactions in breast cancer. J Pineal Res 2005; **38:**217–22.
29 Wiesenberg 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.
30 Winczyk K, Pawlikowski M, Karasek M. Melatonin and RZR/ROR receptor ligand CGP52608 induce apoptosis in the murine colonic cancer. J Pineal Res 2001; **31:**179–82.
31 Winczyk K, Pawlikowski M, Guerrero JM, Karasek M. Possible involvement of the nuclear RZR/RORalpha receptor in the antitumor action of melatonin on murine Colon 38 cancer. Tumor Biol 2002; **23:**298–302.
32 Yuan L, Collins AR, Dai J, Dubocovich ML, Hill SM. MT(1) melatonin receptor overexpression enhances the growth suppressive effect of melatonin in human breast cancer cells.Mol Cell Endocrinol 2002; **192:**147–56.