Oncostatic effects of the indole melatonin and expression of its cytosolic and nuclear receptors in cultured human melanoma cell lines

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Abstract. Melatonin has been shown to have oncostatic effects on malignant melanoma *in vitro* and *in vivo*. We studied the growth suppressive effects of melatonin over a wide range of concentrations in four melanoma cell lines (SBCE2, WM-98, WM-164 and SKMEL-188) representative for different growth stages and phenotype. Melanoma cells were incubated with melatonin 10^{-12}-10^{-3} M, and proliferation and clonogenicity was assessed at 12 h and 14 days, respectively. We also determined the expression of cytosolic quinone oxidoreductases NQO1, NQO2 (known as MT3 receptor) and nuclear receptor RORα by RT-PCR. Melatonin at pharmacological concentrations (10^{-3}-10^{-7} M) suppressed proliferation in all melanoma cell lines. In SKMEL-188 cells cultured in serum-free media, melatonin at low concentrations (10^{-12}-10^{-10} M) also slightly attenuated the proliferation. The effects of pharmacological doses of melatonin were confirmed in the clonogenic assay. Expression of NQO1 was detected in all cell lines, whereas NQO2 and nuclear receptor RORα including its isoform RORα4 were present only in SBCE2, WM-164 and WM-98. Thus, melatonin differentially suppressed proliferation in melanoma cell lines of different behaviour. The intensity of the oncostatic response to melatonin could be related to the cell-line specific pattern of melatonin cellular receptors and cytosolic binding protein expression.

Introduction

Melatonin acts as a hormone (1,2), receptor independent autocrine and paracrine antioxidant (3,4), direct radical scavenger (3,5), immunomodulator (6), antiaging factor (7,8) and anticarcinogen (9) depending on the concentrations (10). Increasing evidence in the field suggests that melatonin acts at many extrapineal sites such as the gastrointestinal tract (11,12), the blood cell system (13), the retina (14), the bone marrow (15,16) and the skin (17-22). In the epidermal keratinocytes, a constitutive and UV-induced melatonin metabolism has recently been described (23). Under physiologic conditions, melatonin participates in the regulation of keratinocyte proliferation (24-26), hair growth (26-31) and melanogenesis (32,33). In conditions of oxidative stress, it acts as an antioxidant (18,34-36) and UV-protective substance (18,22,37,38). These actions may result in protective, proliferative and antiapoptotic effects to ensure the survival of non-neoplastic cells.

In contrast, in malignant processes, melatonin has been shown to inhibit tumor growth by decreasing cell growth through proapoptotic actions. These effects had been observed in tumor cell lines of breast (39), prostate (40) and colon carcinoma (41). Melanoma is an aggressive form of skin cancer associated with high mortality and limited response to therapy once extended beyond the skin (42-44). Melatonin has been shown to suppress cell growth in selected cutaneous and uveal melanoma cell lines of rodent and human origin (32,45-47). Also clinically, there is some evidence for melatonin having potent antineoplastic activity (48-51). The biological activity of melatonin is enhanced by its strong lipophilic property that allows for easy penetration through cell membranes and the possibility of direct intracellular actions. The anticarcinogenic effect seems to be increased through membrane, cytosolic and/or nuclear receptors (45,47,52,53). The membrane receptors, represented by MT1 and MT2, belong to a G-protein coupled receptor super-family which can bind to a variety of G-proteins (54-56) and are differentially expressed in melanoma cell lines (24). Cytosolic NRH:quinone oxidoreductase 2 (NQO2, QR2) possesses melatonin binding sites previously described as MT3 receptor (57), differing in this aspect from NAD(P)H:quinone oxidoreductase 1 (NQO1, QR1) (58). Melatonin nuclear receptors, recently identified in keratinocytes, melanocytes and fibroblasts (18) as well as in hair follicles (28), belong to the retinoid orphan receptor subfamily (RZR/ROR). It was shown that all these human

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Table I. Sequences of primers used for RT-PCR amplifications.

Gene	Primer	Fragment size (bp)	Ref.
RORα common fragment	MZ013 5'-GCTGACATCAGTACAAATGCAG-3' MZ014 5'-GGAAGAGCTCCAGCAGATAACG-3'	320	76
RORα1 forward	MZ016 5'-AAACATGGAGTCAGCTCCG-3'	475	59
RORα2 forward	MZ017 5'-CTCCAAATACTCCATCAGTGTATCC-3'	414	59
RORα3 forward	MZ018 5'-CAACTTGAGCACATAAACTGG-3'	369	59
RORα4/RZRα	MZ015 5'-CGCACCGCGCTTAAATGATG-3'	416	59
Common reverse primer for ROR·1-4	MZ019 5'-CATACAAGCTGTCTCTCTGC-3'		59
NQO1	MZ020 5'-GAGGACCTCCTTCAACTATGCC-3' MZ021 5'-CCTTTGTCATACATGGCAGCG-3'	365	73
NQO2	MZ022 5'-GGAACCCAAGTCTTTCAACGG-3' MZ023 5'-TGGGCTCTTCCTTCCAGATGG-3'	622	73

nuclear receptors are splicing variants [RORα1, RORα2, RORα3, RORα4 (also named RZRα)] of the RORα gene (18,40,59).

Here, we tested melatonin for its potential oncostatic effects by assaying for proliferation and colony growth suppression in four different melanoma cell lines (three amelanotic lines including SBCE2, WM-98 and WM-164 derived from vertical and radial growth phases or metastasis, respectively, and SKMEL-188, which can produce melanin pigment). We also analysed the expression of quinone oxidoreductase 1 and 2 as well as RORα nuclear receptor and its isoforms RORα1, RORα2, RORα3 and RORα4.

Materials and methods

Cell culture, melatonin treatment and [^{3}H]-thymidine incorporation. The human melanoma lines used consisted of SKMEL-188, WM-164, WM-98 and SBCE2. The human hypomelanotic melanoma cell line SKMEL-188 [inducible to differentiate by high concentration of tyrosine (60)] was cultured in Ham's F-10 medium with L-glutamine (Cellgro Mediatech Inc., Herndon, VA). The human amelanotic melanoma lines, established from radial growth phase (SBCE2), vertical growth phase (WM-98) and metastasis (WM-164) (gift of M. Herlyn, Wistar Institute, Philadelphia, PA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose, L-glutamine and pyridoxine hydrochloride (Gibco, Rockville, MD). According to standard protocols, the media contained 5% horse-serum (Invitrogen Corp., Carlsbad, CA), antibiotics (Gibco) and insulin (5 μg/ml; Sigma Chemical Co., St. Louis, MO) in the presence of 5% CO_2 (61). The culture media were changed every other day.

For experiments, melatonin (Sigma) was dissolved in ethanol and further diluted to corresponding test concentrations with PBS (final ethanol concentration <0.2%). The test cells were removed from culture flasks by trypsinization and seeded at a density of 10^3 cells/well in a 96-well microtiter plate (Corning Inc., Corning, NY). Cells were used for experiments after overnight adherence, reaching 70-80% confluency. Before starting melatonin treatment, two different culture conditions were chosen: i) supplemented media were replaced by serum-free media for 24 h to achieve cell cycle synchronisation, and ii) the supplemented media were maintained for 24 h by adding fresh media containing horse-serum. Cells were then incubated with melatonin at graded concentrations from 10^{-12} to 10^{-3} M in fresh media +/- horse-serum corresponding to pretreatment regimen. Parallel controls were incubated with the same media, but without melatonin. After 12 h, melatonin was added again at the same concentrations together with [^{3}H]-thymidine, 1 μCi/ml medium (Amersham Biosciences, Picataway, NY; specific activity 88.0 Ci/mmol). Media containing melatonin and [^{3}H]-thymidine were discarded 12 h later, cells were detached with trypsin and harvested on a glass fiber filter (Packard, Meriden, CA). ^{3}H-radioactivity incorporated in DNA as a marker for cell proliferation was measured with a beta counter (Direct Beta-Counter Matrix 9600, Packard, Meriden, CA).

Colony-forming assay. The SKMEL-188 and SBCE2 melanoma cells were selected for the colony-forming assay. Cells were seeded in Petri dishes at a density of 65 cells/cm^2 and allowed to adhere during overnight incubation at 37˚C and 5% CO_2. Cells were then further incubated with fresh media supplemented with horse-serum (5%) containing melatonin at concentrations of 10^{-11}, 10^{-6}, 10^{-4} and 10^{-3} M for SKMEL-188 melanoma cells and 10^{-12}, 10^{-10}, 10^{-6}, 10^{-4} and 10^{-3} M for SBCE2 melanomas. Cells were cultured for 14 days at 37˚C and 5% CO_2 to allow for colony formation, and media containing melatonin at the test concentrations were changed every 72 h. After 14 days, colonies were fixed with 4% paraformaldehyde overnight at 4˚C, stained with crystalline blue and counted with a colony counter (ARTEK counter, Model 880, DYNEX Technologies Inc., Chantilly, VA).

cDNA preparation and RT-PCR. Total RNA was prepared with a total RNA extraction kit (Qiagen, Valencia, CA)

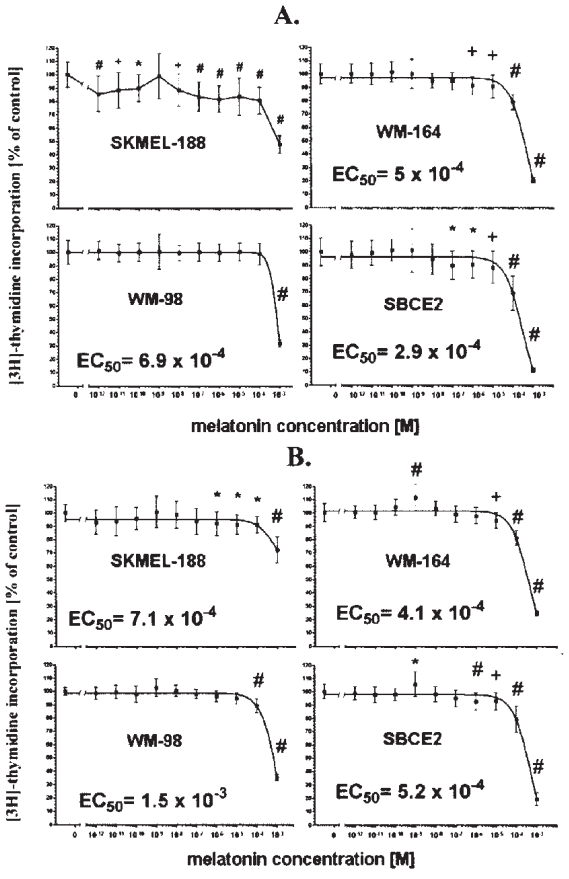

Figure 1. Inhibition of melanoma cell proliferation by melatonin. A, serum-free condition. Melatonin at the concentration of 10^{-3}-10^{-8} M and 10^{-10}-10^{-12} M significantly suppressed [^{3}H]-thymidine incorporation into SKMEL-188 melanoma cells under a starving condition (SKMEL-188). WM-164 melanoma cells were inhibited by melatonin at the concentrations of 10^{-3}-10^{-6} M (WM-164). In WM-98 melanoma cells, suppression was only significant at the melatonin concentration of 10^{-3} M (WM-98). SBCE2 melanoma cells were significantly suppressed at the melatonin concentration of 10^{-3}-10^{-7} M (SBCE2). $^{*}p<0.05$; $^{+}p<0.01$; $^{\#}p<0.001$; mean ± SD. B, serum-supplemented condition. Cell growth in SKMEL-188 was significantly suppressed by melatonin concentrations of 10^{-3}-10^{-6} M (SKMEL-188). In WM-164 melanoma cells, inhibition of proliferation was exerted by melatonin concentrations of 10^{-3}-10^{-5} M (WM-164). WM-98 cells were only significantly suppressed by the concentration of 10^{-3}-10^{-4} M (WM-98), and SBCE2 melanoma cells showed suppression by melatonin at concentrations between 10^{-3} and 10^{-6} M (SBCE2). A slight proliferative effect by melatonin was observed at the concentration of 10^{-9} M in WM-164 and SBC2 melanoma cells. $^{*}p<0.05$; $^{+}p<0.01$; $^{\#}p<0.001$; mean ± SD.

supplemented with an RNase-free DNase set (Qiagen, Valencia, CA). Two μg of total RNA was reverse transcribed with a SuperScript first-strand synthesis system (Applied Biosystems, Foster City, CA). Quality and quantity of all samples were standardized through amplification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA subunit as described previously (18). Primers used for PCR amplification were synthesized by Integrated DNA (Technology Inc., Coralville, IA) and are listed in Table I. PCR reactions were carried out as described previously, using PCR Master Mix (Promega) and 0.4 mM of each primer. Amplification products were separated by agarose gel electrophoresis, visualized by ethidium bromide staining and analyzed with QuantityOne software (Bio-Rad Laboratories, Hercules, CA).

Table II. Inhibition of proliferation in melanoma cell lines exposed to melatonin at the concentration of 10^{-3} M.[a]

Cell line	Serum	[^{3}H]-thymidine incorporation (% of control)	EC_{50}
SKMEL-188	-	~ 50	-
(hypomelanotic phenotype)	+	~ 70	$7.1x10^{-4}$ M
WM-164	-	~ 20	$5.0x10^{-4}$ M
(metastatic stage)	+	~ 25	$4.1x10^{-4}$ M
WM-98	-	~ 30	$6.9x10^{-4}$ M
(vertical growth phase)	+	~ 35	$1.5x10^{-3}$ M
SBCE2	-	~ 10	$2.9x10^{-4}$ M
(radial growth phase)	+	~ 20	$5.2x10^{-4}$ M

[a]The inhibitory effect of melatonin is more pronounced under a serum-free condition (synchronized) compared to a serum-supplemented condition.

Statistical analysis. [^{3}H]-thymidine incorporation into DNA was measured separately for each well and the results from 12 wells per condition were calculated as the mean in a series of repeated independent experiments. Data were analysed with GraphPad Prism Version 4.0 (GraphPad Software Inc., San Diego, CA) using One-way-ANOVA with appropriate *post hoc* testing. Differences were considered significant when $p<0.05$. Numbers of colonies were calculated as the mean of three measurements per condition and analysed as above.

Results

Proliferation assay. A distinct inhibitory effect on melanoma cell proliferation by melatonin was observed in all cell lines. The effect was more pronounced in synchronized cell-cycle growth [serum-free condition (Fig. 1A)] than in cells growing under serum-supplemented conditions (Fig. 1B). The concentration of 10^{-3} M melatonin showed the strongest suppression in all cell lines ($p<0.001$), however, the magnitude of the effect differed across cell lines (Table II and Fig. 1). In SKMEL-188 melanoma cells, melatonin at the concentration of 10^{-3} M suppressed proliferation to 50% of control in a serum-free condition, and to 70% in a serum-supplemented condition. The EC_{50} in the serum-supplemented condition was $7.1x10^{-4}$ M. In the serum-free condition a biphasic dose response was observed (Fig. 1A; SKMEL-188 panel). By comparison, in the WM-164 melanoma cell line (of metastatic origin), melatonin at a pharmacological concentration led to a suppressed viability of 20% (serum-free) and 25% (supplemented) of untreated control. The EC_{50} was 5 and $4.1x10^{-4}$ M, respectively. In WM-98 melanoma cells (vertical growth phase), melatonin at 10^{-3} M showed reduced proliferation of 30% (serum-free) and 35% (supplemented) of

Table III. Melatonin-induced suppression of proliferation in melanoma cell lines over a wide range of concentrations.

Cell line	HS supplement	Melatonin concentration (M)									
		10^{-12}	10^{-11}	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
SKMEL-188	No	+++	++	+	-	++	+++	+++	+++	+++	+++
	Yes	-	-	-	-	-	-	+	+	+	+++
WM-164	No	-	-	-	-	-	-	++	++	+++	+++
	Yes	-	-	-	IP	-	-	-	++	+++	+++
WM-98	No	-	-	-	-	-	-	-	-	-	+++
	Yes	-	-	-	-	-	-	-	-	+++	+++
SBCE2	No	-	-	-	-	-	+	+	++	+++	+++
	Yes	-	-	-	IP	-	-	+++	++	+++	+++

HS, horse-serum. +, suppression of proliferation; level of significance $p<0.05$. ++, suppression of proliferation; level of significance $p<0.01$. +++, suppression of proliferation; level of significance $p<0.001$. -, no effect on proliferation. IP, increased proliferation ($p<0.001$).

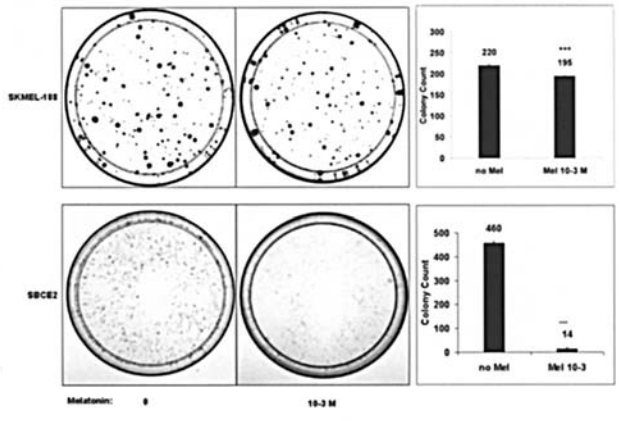

Figure 2. Effect of melatonin on colony forming ability by SKMEL-188 and SBCE2 melanomas. Melatonin at the concentration of 10^{-3} M led to significant suppression of colony formation. Colony counts in SKMEL-188 melanoma cells (upper panel) decreased to 89% of controls; in SBCE2-melanoma cells, suppression was down to 3% of control (lower panel). Experiments were performed in duplicate or triplicate. $^{***}p<0.001$.

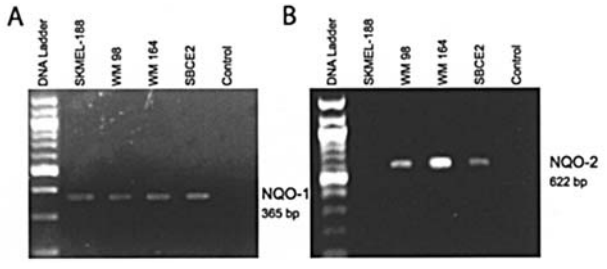

Figure 3. Expression of NQO1 and NQO2 in human melanoma cell lines. NQO1 is present in all tested cell lines; NQO2 was identified in WM-164 > WM-98 > SBCE2 (from highest to lowest expression). Characteristic fragments of NQO1 (A), and NQO2 (B) mRNA were amplified by RT-PCR (73). Last line in each panel represents negative control without cDNA.

control and an EC_{50} of $6.9x10^{-4}$ and $1.5x10^{-3}$ M. The strongest suppression was observed in the SBCE2 melanoma cell line (derived from radial growth phase) with only 10 and 20% cell survival compared to control. The EC_{50} was 2.9 and $5.2x10^{-4}$ M. The sensitivity to melatonin over a wide range of treatment concentrations was however dissociated from the effect on proliferation suppression. Thus, the SKMEL-188 cell line showed significant suppression at both high (10^{-3}-10^{-8} M) and low (10^{-10}-10^{-12} M) melatonin concentrations (Fig. 1A). The suppressive effect of low melatonin concentrations was not seen in the other cell lines (Fig. 1). Surprisingly, two cell lines (WM-164 and SBCE2) showed a slight increase of proliferation after incubation with melatonin at the concentration of 10^{-9} M in serum-supplemented condition (Fig. 1B). An overview of these results is presented in Table III.

Colony-forming assay. The cell lines SKMEL-188 and SBCE2 were used for the clonogenic growth assay. Melatonin at the concentration of 10^{-3} M suppressed colony formation significantly, but again there was a difference in the magnitude of the effect between the two cell lines. While the colony number in melatonin-treated SKMEL-188 melanoma cells showed an 11.4% reduction compared to controls ($p<0.001$; Fig. 2, upper panel), in the SBCE2 melanoma cell line the reduction was 97% ($p<0.001$; Fig. 2, lower panel). Moreover and in correspondence with the thymidine incorporation assay, SKMEL-188 melanoma cells showed significant suppression by melatonin at lower concentrations (10^{-6} and 10^{-11} M), whereas in SBCE2 melanoma cells the effect was not seen at concentrations $<10^{-6}$ M (data not shown).

Expression of quinone oxidoreductase and nuclear melatonin receptor. The quinone oxidoreductase type 1 (NQO1) was expressed in all tested melanoma cell lines (Fig. 3A), and NQO2 was expressed in three of the cell lines with decreasing level of expression (WM-164 > WM-98 > SCBE2) (Fig. 3B). NQO2 mRNA in SKMEL-188 was below the level of detectability. RT-PCR for the nuclear receptor RORα/RZRα and the RORα1, RORα2, RORα3 and RORα4 (RZRα) splicing variants is presented in Fig. 4. The 320-bp cDNA fragment of RORα was detected in three melanoma cell lines, but not in SKMEL-188 (Fig. 4A). Subspecification of receptor isoforms

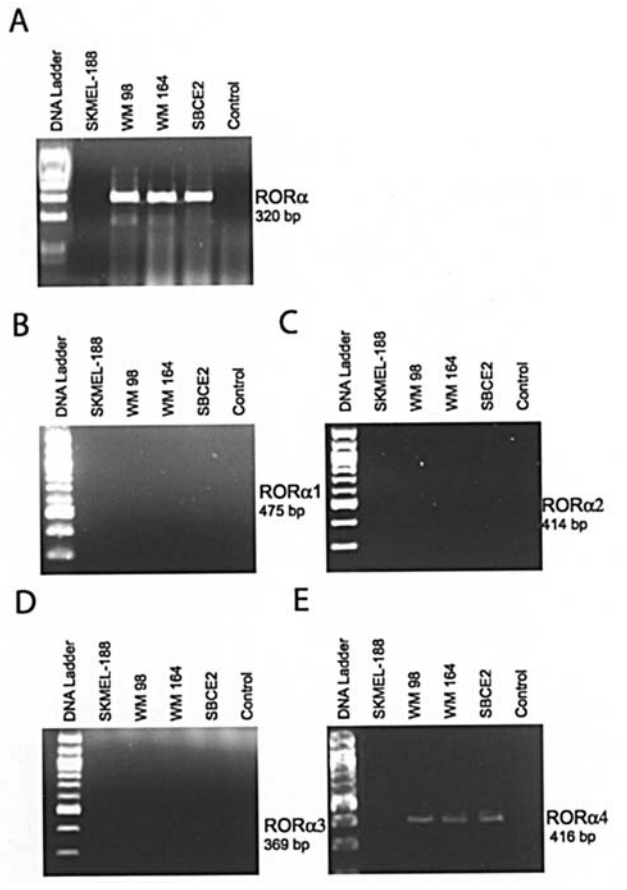

Figure 4. Expression of alternatively spliced nuclear receptors RORα. The common fragment (RORα) and the isoform RORα4 were detected in WM-164, WM-98 and SBCE2 melanoma cell lines, whereas SKMEL-188 did not express the nuclear receptor. Universal primers were used to detect RORα mRNA (A) as described in Materials and methods. Primers for expression of RORα isoforms RORα1 (B), RORα2 (C), RORα3 (D) and RORα4 (RZRα) (E) were studied with a set of primers as published (59). DNA marker was 100-bp ladder except for A, for which GeneRuler 100-bp DNA ladder was used. The last line in each panel represents the negative control without cDNA.

showed RORα4 (RZRα) in WM-98, WM-164 and SCBE2 (Fig. 4E), whereas RORα1, RORα2 and RORα3 were not present in any of the cell lines (Fig. 4B-D). A summary of these results is presented in Table IV.

Discussion

The present study shows distinct oncostatic effects of melatonin at different concentrations in four clinically representative melanoma cell lines under a variety of conditions that included cultivation period (12 h and 14 days), cell line specificity and cell cycle synchronization (free running vs. synchronized). Additionally, the gene expression of quinone oxidoreductase 1 and 2 as well as nuclear receptor RORα and its splicing variants was investigated.

Three cell lines (WM-164, WM-98, SBCE2) were strongly inhibited in their growth by melatonin, whereas SKMEL-188 melanoma cells showed lower suppression. The strongest inhibition by melatonin was observed in melanoma cells that derived from radial growth (SBCE2), putatively representative for the behaviour of early-stage tumor growth. Melanoma cells of vertical growth phase and metastatic tumor origin demonstrated a smaller responsiveness to melatonin.

Differences of growth suppression were also observed in dependency of culture conditions. In SKMEL-188, WM-164 and SBCE2 melanoma cells, the growth suppressing effect of melatonin was stronger when cells had been cultivated in serum-free (synchronized) conditions. Serum-free cultivation leads the cells into a starvation state in which proliferation rate and metabolism are reduced followed by cell cycle synchronization. A stronger suppressive effect of melatonin on cell proliferation in slower growing melanoma cells from earlier passages had been previously noted and ascribed to a loss of receptors regulating the tumor suppressive effect of melatonin (51).

For the clonogenic growth assay, we selected two cell lines exhibiting different responses to melatonin in the thymidine-assay, e.g. SKMEL-188 showing the lowest suppressive effect by melatonin at 10^{-3} M, but the highest sensitivity responding to the concentration of 10^{-10}-10^{-12} M. In contrast, SBCE2 showed the strongest suppression by melatonin (10^{-3} M), but low sensitivity (no effect at concentrations $>10^{-7}$ M). The clonogenic growth assay confirmed the results obtained in proliferation assessment with [^{3}H]-thymidine incorporation. Thus, SKMEL-188 showed lesser inhibitory melatonin effects than SBCE2. Moreover, the sensitivity to melatonin treatment in colony growth increased in SBCE2 melanoma cells, since over a cultivation period of 14 days, the suppression by

Table IV. Expression profile of nuclear receptor RORα and its four isoforms RORα1, RORα2, RORα3 and RORα4 and NQO1 and 2 in melanoma lines.

Cell line	Nuclear receptor RORα	Nuclear receptor splicing variants				Quinone reductases		Housekeeping genes	
		RORα1	RORα2	RORα3	RORα4 (RZRα)	NQO1	NQO2	GAPDH	18S
SKMEL-188	-	-	-	-	-	+	-	+	+
WM 164	+	-	-	-	+	+	+++	+	+
WM 98	+	-	-	-	+	+	++	+	+
SBCE2	+	-	-	-	+	+	+	+	+

melatonin (10^{-3} M) was stronger, to 3% of control as compared to 20% of controls in the [^{3}H]-thymidine incorporation assay. In fact, in both cell lines the effective concentration range on colony growth was comparable with that observed in the [^{3}H]-thymidine incorporation experiments.

The effective concentrations of melatonin (inducing statistically significant suppression) were in the range of 10^{-3} to 10^{-7} for WM-164 and SBCE2 melanoma cell lines and 10^{-3} and 10^{-4} in WM-98. Studies with murine melanoma cells showed proliferation suppressive effects of melatonin at similar concentrations from 10^{-3} to 10^{-6} M (46), and in the human malignant melanoma cell line M6, melatonin effective suppression of forskolin-stimulated cAMP accumulation as a sign for decreased proliferation occurred at 10^{-4}-10^{-9} M (62). A decreased growth rate has been noted in SKMEL-28 human melanoma cells at the minimal effective concentration of 10^{-8} M (63). To the authors knowledge, effective suppression of melanoma cell proliferation by melatonin at concentrations below 10^{-8} M has so far been noted only in rodent melanomas and human uveal melanoma cell lines (10^{-8}-10^{-10} M) (32,64,65) and in PG19 and B16BL6 mouse melanoma (10^{-9}-10^{-11} M) (66). Interestingly, the present study has also shown melatonin effectiveness at concentrations as low as 10^{-12} M in the SKMEL-188 human melanoma cell line. Also of interest is the slight stimulation of growth in two cell lines (WM-164 and SBCE2) at the melatonin concentration of 10^{-9} M, since at the same concentration, a stimulation of 15% was reported in B16 melanoma cells (67).

The expression pattern of membrane and nuclear receptors for melatonin has been found to differentially modify melatonin effects in malignant cells of different origin. For example, studies in the colon 38 carcinoma cell line identified both the nuclear receptor RZR/ROR (RORα) and the cell membrane receptor MT2, but not MT1, as being responsible for the oncostatic effect of melatonin (41,52,68,69). In contrast, the MT1 receptor was responsible for the enhancement of the tumor suppressing effect of melatonin in MCF-7 breast cancer cells (39,53) and in S-91 murine melanoma cells (45). In uveal melanoma cell lines, melatonin and agonists for MT1 and MT2 inhibited melanoma growth, whereas the nuclear receptor was not involved in growth suppression (47). In estrogen-receptor positive breast cancer cells, the expression of RORα was found to clearly contribute to melatonin-induced growth inhibition (70). These observations suggest that the differential expression of membrane and nuclear receptors defines a specific cell-type related phenotype, which may be the main determinant of specific responses to melatonin.

The strongest proliferation suppression in the present study was found in the melanoma cell lines WM-164, WM-98 and SBCE2 that express the nuclear receptor RORα and its specific isoform RORα4. These results cannot be compared with the literature since to the authors' knowledge, this is the first report on RORα nuclear receptor and its four splicing variants RORα1, RORα2, RORα3 and RORα4 in human malignant melanoma cell lines. Expression of RORα nuclear receptor, however, was previously demonstrated in skin cells [including normal and immortalized melanocytes and keratinocytes and dermal fibroblasts (18)] and in hair follicles (28). Whether the present observation of differential melatonin receptor expression in malignant cells has a causal correlation with melatonin responsiveness, will be a subject of further testing with nuclear receptor antagonists and/or RORα$^{-/-}$ receptor cell lines. The strong proliferation suppression induced by high concentrations of melatonin in RORα positive melanoma cells may be explained by intracellular diffusion into the nuclear compartment with building high melatonin intranuclear levels that effectively bind the nuclear receptor. Thus, it can be speculated that activation of nuclear receptor RORα executes a proapoptotic program, which leads to cell death in the majority of the cell population. Interestingly, lines expressing nuclear receptors showed stronger growth inhibition. In addition, expression of membrane receptor MT2 as observed in SKMEL-188 melanoma cells in previous studies (24) seems to contribute to the high sensitivity of this cell line to inhibition by melatonin at low concentrations (10^{-12}-10^{-10} M), but also to the small magnitude of the suppressing effect.

The enzymes NAD(P)H:quinone oxidoreductase 1 (NQO1, QR1) and the NRH:quinone oxidoreductase 2 (NQO2, QR2), which is identical with the melatonin binding site MT3 (71,72), have been previously detected in normal and immortalized melanocytes and keratinocytes as well as in adult dermal fibroblasts (18). In the present study we extended this investigation to melanoma cell lines. Whereas NQO1 was expressed in all tested cell lines, NQO2 expression was limited to WM164, WM-98 and SBCE2 cell lines. NQO1 is ubiquitously expressed in physiological and pathological conditions. In malignant conditions, Vella *et al* found NQO1 in solid tumors of liver, thyroid, adrenal, breast, colon, and non-small cell lung cancers (58). In contrast, NQO2 expression is quite variable and dependent, among others, on the species tested. High gene expression, for example, is detected in the skeletal muscle of humans, but not in mouse muscle (58). NQO2 was also found in relatively high levels in normal human liver, while expression of NQO1 was very low, but reverse expression was found in malignant tissues (73). Even though to date the biological role of NQO2 is poorly understood, there are studies showing evidence for its involvement in skin carcinogenesis. The most prominent example is that NQO2 knockout mice are far more prone to develop skin tumors after the topical application of carcinogens (74). More recently, inhibition of melanoma cell growth and clonogenicity was correlated with upregulation of NQO2 and p53 (75). In this context, the parallel inhibition of cell growth induced by melatonin, the natural ligand of NQO2, suggests that expression of NQO2 might be another factor determining the oncostatic effects of melatonin.

In conclusion, there may be dynamic correlations between melatonin receptor expression and its oncostatic effects. Since there is also heterogenity across different melanoma cell lines, more work is needed to fully characterize the factors determining dependency of dynamic fluctuation over time and tumor behavior. Clarification of this area will allow extension of our results to the clinically encountered forms of malignant melanoma. Moreover, as a concept for optimizing anticarcinogenic treatment with melatonin, future *in vitro* and *in vivo* studies may use combinations of selective agonists for RORα, MT2 receptor and the cytosolic NQO2 melatonin binding site to increase sensitivity and response to treatment.

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