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ORIGINAL ARTICLE

The influence of nano-TiO_2 on metabolic activity, cytotoxicity and ABCB5 mRNA expression in WM-266-4 human metastatic melanoma cell line

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Summary

Purpose: *There is no clear evidence on whether sunscreens and personal care products containing UV-filters like titanium dioxide (TiO_2) are protective against or may be a contributing factor in melanoma development. Extensive studies have shown that TiO_2 can cause cell toxicity under in vitro and in vivo conditions. The transmembrane protein ABCB5 is closely linked to tumorigenicity, progression and disease recurrence of diverse human malignancies, including melanoma. Accordingly, the aim of the present study was to investigate in vitro any potential influence of nanosized TiO_2 (nano-TiO_2) on metastatic melanoma cells' metabolic activity, cytotoxicity and ABCB5 mRNA expression.*

Methods: *The human metastatic melanoma cell line WM-266-4 (ATCC) was used to obtain dose- and time-dependent responses. We used the MTT and LDH assays to measure metabolic activity of selected cells and cytotoxicity. Real-time quantitative PCR (RT-qPCR) was performed and gene expression ratios were calculated for the target (ABCB5) and the reference (LDHA) gene. Standard statistical tests were used for analysis in SPSS and Excell.*

Results: *Our results suggest decreased metastatic melanoma cells' metabolic activity, increased cytotoxicity and increased ABCB5 mRNA expression after 24 and 48 hrs as compared to control (untreated) cells ($p<0.05$). Thus, we showed that nano-TiO_2 might influence cells' invasiveness and aggressiveness.*

Conclusion: *We show for the first time that ABCB5 expression in metastatic melanoma cells might be affected by nano-TiO_2 exposure. In addition, nano-TiO_2 as a sunscreen ingredient might play a role in metastatic melanoma progression.*

Key words: *ABCB5, melanoma, nanoparticles, titanium dioxide, WM-266-4*

Introduction

Excessive exposure to ultraviolet (UV) radiation contributes to the development of melanoma [1-3]. Over time, sun exposure is known to cause DNA damage and systemic immunosuppression, which are factors for carcinogenesis [2]. Current methods of photoprotection include sun avoidance, seeking shade, use of protective clothing and the application of sunscreen [4]. Of these, today, topical application of sunscreens, containing UV-filters, remain the most prevalent protection strategy against adverse effects of UV radiation [1].

Evidently, use of sunscreens is effective in prevention of sunburns in various models [5]. Although exposure to UV radiation is the only known modifiable cause of melanoma, evidence for the protective effects of sunscreens in melanoma pre-

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vention is less conclusive [5]. And even though the utilization of sunscreens with UV-filters is increasing, the incidence of the malignant disorder for which sunscreens should protect, melanoma, is rapidly increasing [5]. Controversy has also developed regarding the possibility of adverse biological effects from various ingredients in sunscreens [4].

Titanium dioxide has for decades been approved for use in sunscreens as inorganic physical sun blocker [6]. TiO_2 provides protection in the UV-A and UV-B ranges which makes it a very effective sunscreen for use in cosmetic and skin care products [7]. To overcome the cosmetically undesired opaqueness of these sunscreens, microsized TiO_2 has been increasingly replaced by nanosized TiO_2 (<100 nm) [6]. It is estimated that worldwide use of nano-TiO_2 in sunscreens is around 1000 tons per year [8]. Inclusion of nano-TiO_2 UV-filter into sunscreens has raised interesting questions regarding the potential for dermal penetration, systemic absorption and subsequent toxicity [4]. Several *in vitro* and *in vivo* studies using both animal and human skin have shown that penetration of nanosized TiO_2 is limited to the stratum corneum, thereby precluding systemic absorption [9-12]. On the other hand, several *in vitro* studies have shown the capacity of TiO_2 nanoparticles to induce oxidative stress resulting in cytotoxicity, cell damage, reactive oxygen species (ROS) production and genotoxicity in various cell lines including dermal cells [8,11,13-17]. This can lead to mutations, DNA damage which, in turn, can induce skin aging, immunosuppression and even skin cancer development [15-17].

Melanoma is the malignancy responsible for the highest incidence of deaths from skin cancer [18]. Genetic and environmental factors such as UV radiation can cause transformation of skin melanocytes into a tumorigenic melanoma [18]. Metastatic melanoma is a highly aggressive tumor resistant to all current conventional systemic therapies [18]; thus, methods to analyze specific markers that drive tumorigenic growth, aggressiveness and resistance should address these biological activities.

Of particular interest is a human ABC transporter protein (ABCB5), a previously described cell-surface marker for melanoma-initiating cells, and the third member of the human P-gp family next to its structural paralogs ABCB1 and ABCB4 [19,20]. ABCB5 expression is closely linked to tumorigenic potential and metastatic disease progression of diverse human malignancies (colorectal cancer, hepatocellular carcinoma, breast cancer) including melanoma and regulates many biological activities such as tumor progression, malignant recurrence and drug resistance [19,21-27].

In melanoma, ABCB5 expressing cells are endowed with self-renewal, differentiation and tumorigenicity abilities [24,27-29]. Cells expressing ABCB5 have survival advantage over the bulk of tumor cells that form the tumor mass [28,30]. This leads to a clinical relapse in patients with supposedly cured melanoma even some years after treatment with chemotherapy and radiotherapy [28].

In the light of relevant data available from the literature, there are no available *in vitro* or *in vivo* studies that had investigated the effects of nano-TiO_2 on metastatic melanoma cells. In addition, whether nano-TiO_2 has any influence on ABCB5 mRNA expression in metastatic melanoma cells, has not yet been experimentally tested to date.

This study aimed to discuss the influence of nano-TiO_2 as a sunscreen ingredient on the metabolic activity, cytotoxicity and ABCB5 mRNA expression in a selected metastatic melanoma cell line. We hypothesized that nano-TiO_2 increases the metabolic activity, cytotoxicity and ABCB5 mRNA expression in metastatic melanoma cell line. Results suggest decreased metastatic melanoma cells' metabolic activity, increased cytotoxicity and increased ABCB5 mRNA expression after 24 and 48 hrs as compared to control cells.

Methods

Metastatic melanoma cells and culture methods

The WM-266-4 (ATCC® CRL1676™) human metastatic melanoma cell line, derived from a 58-year-old woman, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in 75 cm^2 flasks, in complete medium containing Eagle's Minimum Essential Medium (EMEM, ATCC® 30-2003™) supplemented with 10% fetal bovine serum (FBS) (ATCC® 30-2021™) and 0.02% MycoZap Plus-CL (Lonza, Portsmouth, NH, USA), and incubated at 37°C with 5% CO_2 in a ≥90% humidified atmosphere. Complete medium was replaced every 48 hrs. When the culture became ~80% confluent, the cells were subcultivated by trypsinization using Detach kit (catalog number C-41220, PromoCell, Heidelberg, Germany, EU) and replated.

Preparation of nano-TiO_2

TiO_2, rutile, nanopowder with <100 nm particle size, was purchased from Sigma - Aldrich, USA (Cat. No. 1317-80-2). A 10 mg/mL stock solution of nano-TiO_2 was prepared and diluted with the complete medium to form the following experimental nano-TiO_2 concentrations: 250, 100, 20, 10, and 1 μg/mL, which were applied to the cells attached to the wells of a 6-well or 24-well culture plates.

Cell metabolic activity testing (MTT assay)

For the MTT assay, WM-266-4 metastatic melanoma cells were seeded in appropriate density in 24-well culture plates and incubated overnight in complete

medium to allow cell attachment. After cell attachment, selected concentrations of nano-TiO_2 were added and further cultured for 8, 24, 48 and 120 hrs. At the end of each incubation period, the MTT Colorimetric Cell Viability Kit IV (PromoKine, Heidelberg, Germany, EU) was performed, strictly following the manufacturer's instructions. The absorbance signal was measured at 570 nm and background absorbance at 630 nm (Infinite® 200 PRO spectrophotometer, Tecan). The percentage of the cell's metabolic activity was calculated with the following equation:

$(A_{570}-A_{630})$ test sample value / $(A_{570}-A_{630})$ control value ×100.

Cell cytotoxicity testing (LDH assay)

The cells were plated at appropriate density in 24-well culture plates and incubated overnight in complete medium to allow cell attachment. The WM-266-4 cells were exposed to five selected nano-TiO_2 concentrations for 48 and 120 hrs to evaluate its cytotoxicity. After each incubation, the media were transferred into a fresh 96-well plate and analyzed for LDH release. The cytotoxicity assay using LDH Cytotoxicity Kit II (PromoKine, Heidelberg, Germany, EU) was performed, strictly following the manufacturer's instructions. The final absorbance signal was obtained by subtracting background absorbance at 650 nm from absorbance signal at 450 nm (Infinite® 200 PRO spectrophotometer, Tecan). The percentage of the cytotoxicity was calculated with the following equation:

$((A_{\text{Test Sample}} - A_{\text{Low Control}}) / (A_{\text{High Control}} - A_{\text{Low Control}})) \times 100$:

where the cells (in EMEM) with the cell lysis solution represented the high control and the cells (in EMEM) the low control.

RNA isolation and cDNA synthesis

The melanoma cells were seeded at appropriate density into 6-well culture plates and incubated overnight in complete medium. Afterwards, the cells were treated with six different concentrations of nano-TiO_2 (250, 125, 50, 20, 10, 1 μg/mL) and incubated for 2, 24, 48 and 120 hrs. Total RNA was isolated from the treated samples and control cells using High Pure RNA Isolation Kit (Cat. No. 11 828 665 001, Roche, Basel, Switzerland) following the manufacturer's protocol. Concentration and purity of the RNA were checked by 260/280 nm ratio using the Thermo Scientific NanoDrop 2000c spectrophotometer.

Reverse transcription into cDNA was performed with Transcriptor Universal cDNA Master Kit (Cat. No. 05 893 151 001, Roche, Basel, Switzerland) using 270 ng of the template RNA.

Real-time quantitative PCR

RT-qPCR was used to evaluate the expression profile of the *ABCB5* gene in control cells as well as in treated samples. It was performed on a LightCycler480 system II in a 20 μl reaction mix containing 100 ng cDNA, 2x LC480 Probes Master and 40x RT Ready Designer/Catalog assay. All reagents for RT-qPCR were purchased from Roche, Basel, Switzerland.

LDHA was chosen as an appropriate endogenous control, as under our experimental conditions its expression was most stable among all tested endogenous controls. PCR amplification efficiency for ABCB5 and LDHA was determined from a standard curve using template dilutions of 1:1, 1:10, 1:100 and 1:1000 in the same PCR conditions.

The protocol included an initial pre-incubation step for denaturation of the template cDNA at 95°C for 10 min, followed by 40 cycles at 95°C denaturation for 10 s, 60°C annealing for 30 s and 72°C elongation for 1 s and the the final step 40°C cooling for 30 s. All samples were run in triplicate. We calculated the mRNA levels by the standard $2^{-\Delta\Delta Ct}$ cycle threshold method normalized to the reference *LDHA* gene.

Cell morphology

Changes in cell morphology were recorded with a digital camera (DFC365 FX Leica, Buffalo Grove, IL, USA) attached to an inverted microscope (DMI6000B, Leica).

Statistics

All the data were plotted using GraphPad and presented as the means ± SEM (standard error of the mean) or as means with a 95% CI (confidence intervals) where indicated. One-way ANOVA followed by a *post-hoc* Bonferroni test were used to determine the significance for MTT and LDH test results. The Student t-test was used to examine the statistical significance of ABCB5 mRNA expression between treated samples and control cells. SPSS 20 (IBM) and Excel software were used for analysis. The level of significance was set at $p<0.05$ (marked with *).

Results

Influence of nano-TiO_2 on cells' metabolic activity of metastatic melanoma cells

Dose- and time-dependent responses on WM-266-4 cells metabolic activity were evaluated using the MTT assay. The obtained results show the percentage of metabolic activity as a function of the different nano-TiO_2 concentrations over time. Cells' metabolic activity percentage was calculated relative to control cells at each incubation time.

An overall reduction of cells' metabolic activity was observed (Figure 1). *Post-hoc* Bonferroni test revealed a significant fall ($p<0.05$) in the metabolic activity after 8 hrs (57.4% ± 3.6%) and 24 hrs (50.7% ± 1.1%) relative to the control cells at the same incubation times, regardless of nano-TiO_2 concentration. Then, a significant increase ($p<0.05$) in the metabolic activity between 8 and 48 hrs was observed within each concentration, but still below the control value (with the mean 92.5% ± 1.8%). A significant decrease of metabolic activity ($p<0.05$) after 120 hrs was observed at concentrations of 100 and 250 μg/mL to mean 47.5% ± 2.5% and 41.5% ± 0.5% relative to the control cells, respectively (Figure 1). Thus, our results show that nano-TiO_2 might be toxic to the metastatic WM-266-4 melanoma cells.

Cytotoxicity evaluation of nano-TiO_2 on metastatic melanoma cells

We aimed to understand if any potential change in the cells' metabolic activity would be related to the nano-TiO_2 cytotoxicity. LDH assay was employed to estimate the cytotoxicity of nano-TiO_2 to WM-266-4 cells *in vitro*. Our results indicate that with the increasing concentrations of nano-TiO_2, the cytotoxicity increase, suggesting a classic dose-dependent behavior *in vitro*. The time-dependent behavior revealed that the longer the WM-266-4 was exposed to nano-TiO_2, the greater the cytotoxic response (Table 1).

Microscopic observation

We noted that the filter was evenly distributed over the areas without cells, but tended to aggregate and arrange into spherical assemblies at the cell surface (Figure 2A,B). The question remains whether nano-TiO_2 enters the cell or remains adhered to them (Figure 2C,D). As shown in Figure 3 A-D, it appears that even the highest concentrations of nano-TiO_2 would not cause major morphological changes than that of control cells. Only a few spindle-shaped cells were observed after 120 hrs among the control cells when compared to 48 hrs (Figure 3B,D).

RNA quality and RT-qPCR analysis for ABCB5

Total RNA was isolated and examined to determine its concentration and purity. The extracted RNA 260/280 nm absorption ratio ranged from 2.00 to 2.07 (mean 2.05 ± 0.004), reflecting pure and protein-free isolated RNA.

We analyzed ABCB5 mRNA expression in WM-266-4 cell line using RT-qPCR. The results indicate that ABCB5 expression level in WM-266-4 cells treated with nano-TiO_2 was significantly higher after 24 and 48 hrs than that after 2 hrs relative to control cells, regardless of nano-TiO_2 concentration (Figure 4). On the other hand, our results showed that ABCB5 expression level was significantly lower after 120 hrs when compared to control cells, regardless of nano-TiO_2 concentration. Taken together, these results clearly showed that nano-TiO_2 influences the expression of ABCB5 in metastatic melanoma cells.

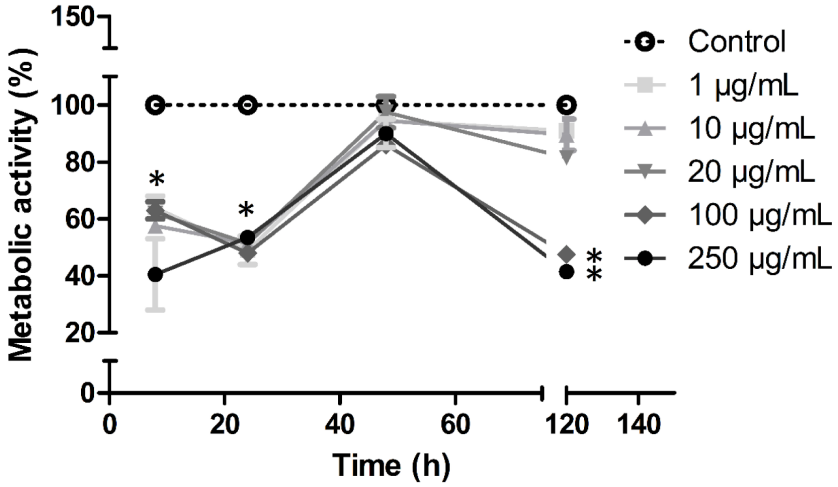

Figure 1. Nano-TiO_2 decreases metabolic activity of metastatic melanoma cells. MTT assay was used for evaluation of cells' metabolic activity. Cells were treated with different concentrations of nano-TiO_2 and incubated for 8, 24, 48 and 120 hrs. Results are shown as mean ± SEM. Treated samples significantly different from control cells [by one-way ANOVA $p<0.05$ followed by *Post-hoc* Bonferroni test] are shown by *$p<0.05$.

Table 1. The cytotoxicity increases with the increasing concentrations and longer time of exposure to nano-TiO_2. WM-266-4 cells were treated with different nano-TiO_2 concentrations and incubated for 48 120 hrs. Data represent means±SEM compared to control cells

	Time	*Different nano-TiO2 concentrations (µg/mL)*				
		1	*10*	*20*	*100*	*250*
Cytotoxicity (%)	0 h	0	0	0	0	0
	48 h	0.7 ± 0.2	0.7 ± 0.2	1.7 ± 0.2	8.3 ± 0.3	10.2 ± 0.1
	120 h	0.5 ± 0.0	5.2 ± 0.8	8.0 ± 1.5	17.8 ± 1.2	23.4 ± 0.3

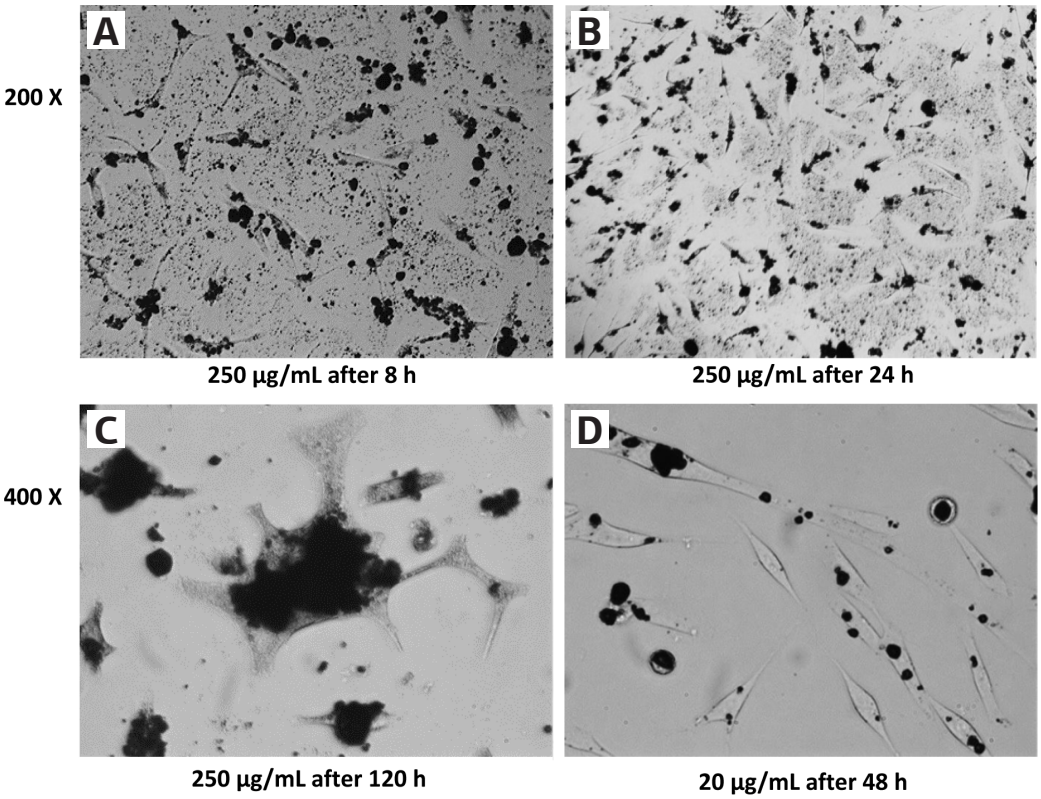

Figure 2. The filter is evenly distributed over the areas without cells, but aggregate at the cell surface **(A,B)**. The question remains whether nano-TiO_2 enters the cell or remains adhered to them **(C,D)**. Representative bright field images of WM-266-4 cells treated with different nano-TiO_2 concentrations. Magnification 200× and 400×.

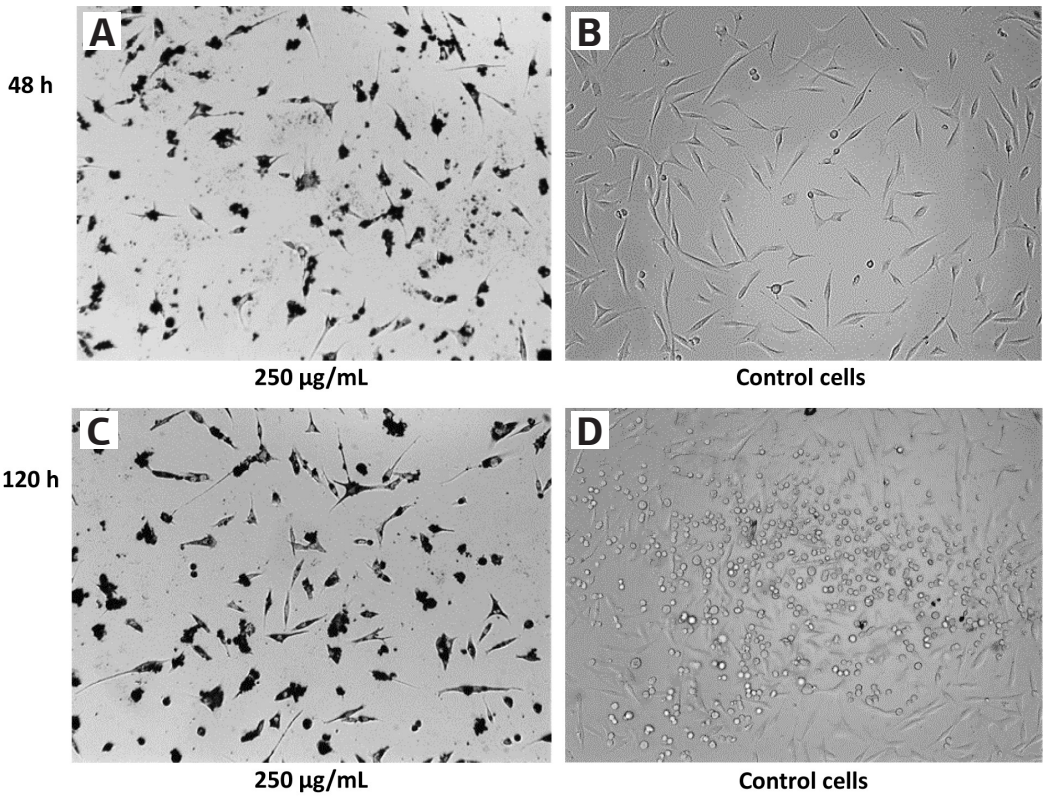

Figure 3. The highest concentrations would not cause major morphological changes or increased cell death in comparison to control cells **(A-D)**. Representative bright field images of WM-266-4 cells treated with different nano-TiO_2 concentrations after 48 and 120 hrs. Note only few spindle-shaped cells after 120 hrs among control cells when compared to 48 hrs **(B,D)**. All images are at 100× magnification.

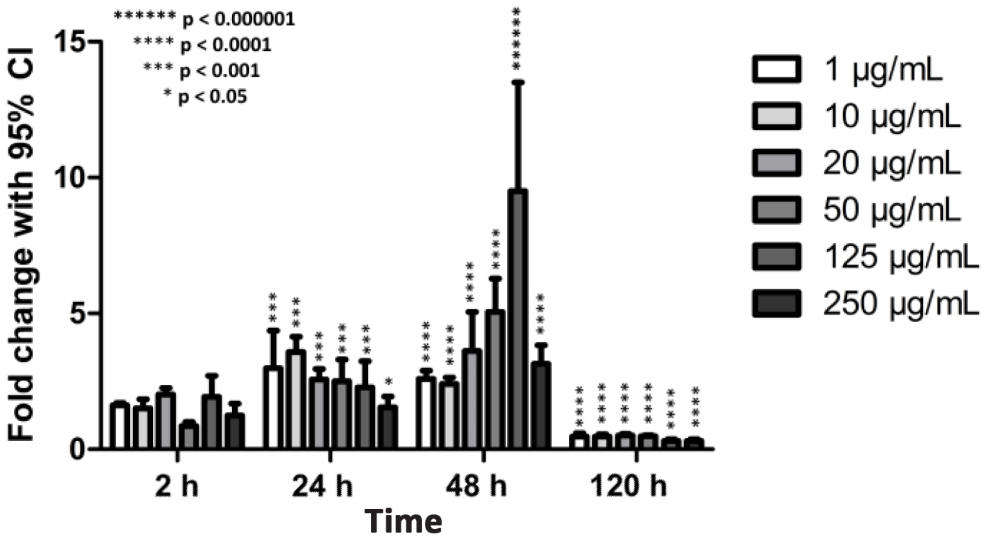

Figure 4. Nano-TiO_2 significantly increases ABCB5 mRNA expression after 24 and 48 hrs in treated cells compared to the expression in control cells. RT-qPCR was used to evaluate the expression profile of the ABCB5 gene and mRNA levels were normalized to the reference LDHA gene. The bars represent means with 95% CI. Treated samples significantly different from control cells (by Student t-test p<0.05) are shown by *.

Discussion

Metastatic melanoma is the most aggressive skin cancer and ABCB5 expression is associated with its aggressiveness. TiO_2, as one of the most widely used nanomaterials in everyday life, has emerged as a potential killer of cancer cells [13,15]. Until now, no study has been carried out showing the effects of nano-TiO_2 on the melanoma cells neither on ABCB5 expression. In this study, we investigated the effects of nano-TiO_2 on the metabolic activity, cytotoxicity, and ABCB5 mRNA expression in metastatic melanoma cells. Our selected concentrations of nano-TiO_2 were similar to those used in previous nano-TiO_2 penetration studies and related keratinocyte toxicity [31-33]. We were interested in any basic effects of nano-TiO_2 on WM-266-4 cells and hence the UV-filter was not exposed to UV light.

As assessed by a colorimetric MTT assay, nano-TiO_2 significantly decreased the metabolic activity in WM-266-4 cells. It was also important to observe that in all the treatment protocols (concentrations, durations) the decrease in metabolic activity was always below the control values. The overall decrease of metabolic activity suggests that the nano-TiO_2 could aggregate in certain intracellular regions, which suggests a ROS-mediated cytotoxic potential of nano-TiO_2 [31,33,34]. This would explain our microscopic observations which seems to show that nano-TiO_2 aggregate at the cell surface (Figure 2A-D) and could be taken up by endocytosis as shown in the previous studies done on keratinocytes and human fibroblasts [34,35]. Based on microscopic observations, we assumed that the cells attract the filter. The question remains whether nano-TiO_2 enters the cell or remains adhered to them (Figure 2C,D). It would be reasonable to examine in further studies what this means at the level of molecular changes in metastatic melanoma cells. To confirm our MTT results and microscopic observations we performed the LDH cytotoxicity test.

Our results indicate that the effect of nano-TiO_2 on cell cytotoxicity is dose- and time-dependent. Table 1 shows the longer the WM-266-4 cells were exposed to nano-TiO_2, the greater the cytotoxic response. The exposure time was truncated at 120 hrs. After 48 hrs, the cell media already lack sufficient nutrients for cells in culture to remain vital, following the manufacturer's instructions. Therefore, the increased cytotoxicity after 120 hrs could be importantly influenced by the lack of nutrients. Overall, the cytotoxicity in culture was low (Table 1) and besides, the microscopic observations confirm this LDH results. As shown in Figure 4, it appears that even the highest concentrations of nano-TiO_2 would not cause major morphological changes than that of control cells. Interestingly, we did the same study on the same cell line, but with another UV-filter, octocrylene, where we found out microscopically significant changes in cell morphology, cell death and indications of cannibalistic activity [36].

The low cytotoxicity of nano-TiO_2 on WM-266-4 cells could be attributed to the fact that these cells are generally very resistant since they contain ABCB5 protein that appears to play a major role in the cell aggressiveness, resistance and survival advantage [13,28]. Moreover, nano-TiO_2 might stimulate the expression of ABCB5 at the cell surface and

induce a substantial increase in ABCB5 expressing cells. For this purpose, we used RT-qPCR to investigate the expression of ABCB5 in metastatic melanoma cells treated with nano-TiO_2.

Our results indicate that despite the inhibitory effects of nano-TiO_2 on the cells' metabolic activity, the ABCB5 expression was significantly higher after 24 and 48 hrs in all tested concentrations relative to control cells. Based on ABCB5 mRNA expression it seems that nano-TiO_2 stimulates ABCB5 expression in metastatic melanoma cells and it could be speculated that the surviving cells are more invasive, aggressive and resistant than the starting melanoma cells or cells that represent the control, due to the characteristic of the ABCB5 protein. It was shown that ABCB5 positive melanoma cells have a greater ability of tumor formation as well as greater ability to self-recovery, differentiation and metastasis, compared to cells that are negative for this marker [28,37,38]. After 120 hrs, the ABCB5 expression in the surviving cells was significantly decreased in all tested nano-TiO_2 concentrations relative to control cells. As mentioned previously culture media should be replaced every 48 hrs to maintain optimum culture conditions. Therefore, ABCB5 expression could be importantly influenced by the age of the medium in condition of low nutrient supply, showing significant decrease in ABCB5 expression after 120 hrs. Also, our microscopic observations were consistent with this finding, as only a few spindle-shaped cells were observed after 120 hrs among the control cells when compared to 48 hrs (Figure 3B,D).

The supposed increased aggressiveness of the cells with increased ABCB5 mRNA expression after 24 and 48 hrs was supported with our microscopic observations which seem to show that nano-TiO_2 might penetrate the cell and as such stimulate cells invasiveness and aggressiveness. Further research and testing should be done to clarify the details of interaction between WM-266-4 cells and nano-TiO_2 at microscopic level.

The effects of nano-TiO_2 have so far been tested only on other cell lines. For the first time, this study presents the effects of nano-TiO_2 on a metastatic melanoma cell line. In practice, our results obtained on a metastatic melanoma cell line suggest that patients with diagnosed metastatic melanoma should avoid the usage of products containing nano-TiO_2, because this could increase progression and recurrence of their disease. For more relevant results, future studies need to confirm our results with ABCB5 protein expression. Additionally, the safety profile of nano-TiO_2 must be established on healthy melanocytes (primary culture) and melanoma cells from primary site before any definitive conclusions. Another point for future research would also be to test the effect of nano-TiO_2 on other potential markers for melanoma cancer cells, i.e. NGFR, ALDH, CD271, RANK, MCSP, MCA [26,27,39,40].

Nevertheless, we believe that the results of our study serve as a starting point for further molecular studies and research with this and other UV-filters, which will clarify and assess the potential clinical applications for melanoma.

Ethical Adherence

All the research work was done according to highest biomedical ethical principles and standards.

This work has partially been presented at two symposia:

- 21st Scientific Symposium of the Austrian Pharmacological Society. Joint Meeting with the British Pharmacological Society and the Pharmacological Societies of Croatia, Serbia and Slovenia. Graz, September 2015
- ELIXIR-SI Launch & 11th CFGBC Symposium: "Data for Life". Ljubljana, September 2016

Conflict of interests

The authors declare no conflict of interests.

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