Mitochondrial function is controlled by melatonin and its metabolites in vitro in human melanoma cells

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Abstract
Melanoma is a leading cause of cancer deaths worldwide. Although immunotherapy has revolutionized the treatment for some patients, resistance towards therapy and unwanted side effects remain a problem for numerous individuals. Broad anti-cancer activities of melatonin are recognized; however, additional investigations still need to be elucidated. Herein, using various human melanoma cell models, we explore in vitro the new insights into the regulation of melanoma by melatonin and its metabolites which possess, on the other side, high safety profiles and biological meaningful. In this study, using melanotic (MNT-1) and amelanotic (A375, G361, Sk-Mel-28) melanoma cell lines, the comparative oncostatic responses, the impact on melanin content (for melanotic MNT-1 melanoma cells) as well as the mitochondrial function controlled by melatonin, its precursor (serotonin), a kynuric (N1-acetyl-N2-formyl-5-methoxykynuramine, AFMK) and indolic pathway (6-hydroxymelatonin, 6(OH) MEL and 5-methoxytryptamine, 5-MT) metabolites were assessed. Namely, significant disturbances were observed in bioenergetics as follows: (i) uncoupling of oxidative phosphorylation (OXPHOS), (ii) attenuation of glycolysis, (iii) dissipation of mitochondrial transmembrane potential (mtΔΨ) accompanied by (iv) massive generation of reactive oxygen species (ROS), and (v) decrease of glucose uptake. Collectively, these results together with previously published reports provide a new biological potential and make an imperative to consider using melatonin or its metabolites for complementary future treatments of melanoma-affected patients; however, these associations should be additionally investigated in clinical setting.

KEYWORDS
extracellular acidification rate, glucose uptake, melanoma cells, metabolites of melatonin, mitochondrial function, oxygen consumption rate, transmission electron microscopy

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Melatonin (N-acetyl-5-methoxytryptamine) is a mediator secreted by the pineal gland. In animals and humans, it is not only an endogenous synchronizer of seasonal biorhythms and sleep-wake cycle but it also regulates immune and endocrine functions. It has anti-cancer activities and possesses strong anti-oxidative, anti-inflammatory or anti-apoptotic properties against the nervous system and peripheral organs, in immune and endocrine functions. It has anti-cancer activities7 and possesses strong anti-oxidative, anti-inflammatory or anti-apoptotic properties against environmental insults.

Melatonin is synthesized via multistage process from tryptophan and its subsequent conversion into N-acetylsertotonin (NAS) from serotonin resulting melatonin formation. It is also produced in the nervous system and peripheral organs, including rodent23,24 and human skin.25,26 Melatonin is metabolized to 6-hydroxymelatonin (6(OH)MEL) and 5-methoxytryptamine (5-MT) via the indolic pathway or to N-acetyl-N-formyl-5-methoxykynuramine (AFMK) and N-acetyl-5-methoxykynuramine (AMK) via the kynuric pathway. Melatonin has been reported as an amphiphilic substance which easily crosses intracellular membranes reaching other organelles including mitochondria. It dynamically interacts with lipid bilayers, and it stabilizes the mitochondrial inner membrane attenuating oxidative damage under stress conditions.4,8-10,12,13,15,18,30-33 Thus, melatonin has been described as an effective modulator of mitochondrial integrity and physiology.

To date, high number of investigations focused on the impact of melatonin in regulation of body homeostasis. Epidemiological studies have reported a clear association between disturbed circadian rhythms among night-shift workers, including nurses or security guards with increased incidences of breast and prostate cancers, respectively. Furthermore, melatonin plays the key regulatory role in skin functions. Despite the maintenance of the epidermal homeostasis, there is lack of knowledge in terms of biological impact of melatonin’s metabolites on melanoma cells. Herein, we deliberately focussed on the mitochondrial function and melanin content in human melanoma cells in vitro which have not been tested so far in presence of melatonin, serotonin (precursor of melatonin) and its selected metabolites (AFMK, 6(OH)MEL or 5-MT).

Nowadays, melanoma is one of the deadliest cancers in the world with an increasing incidence. It represents the most rapidly rising malignancy in the Caucasian population. It is derived from genetically altered melanocytes following complex interactions between genetic, constitutive and environmental factors. In cutaneous melanoma, BRAF mutations are more common on intermittently sun-exposed skin. Thus, the BRAFV600E (50% of melanomas harbour BRAF mutations) leads to constitutive activation of downstream kinases of the mitogen-activated protein kinase (MAPK) signalling cascade in melanomas.

For now, obtained results within this study together with our recent reports enhance the biological importance of these compounds, provide the new insights into biology of melanoma and pave the path to consider melatonin, its metabolites or serotonin for future treatment of melanoma patients in combination with currently available targeted therapies.
up to 72 hours. Ethanol itself at the concentration of 0.2% (control cells) was not toxic for the cells. All cell lines in the logarithmic growth phase were used in all experiments, while 80-90% monolayers of confluent cells were harvested with a mixture of 0.05% trypsin-EDTA solution.

2.3 | Cell viability assay

Cells seeded in 96-well plates at the density of $0.15 \times 10^5$ cells/well were incubated with tested substances until desired time end point. Proliferation/growth rate was developed using the MTT assay along the previously described procedure.  MTT (5 mg/mL in 1 x PBS) was prepared in culture medium (the final dilution, 1:10), 100 μL of assay reagent was added to each well, and cells were subsequently incubated for 3 hours in a humidified atmosphere of 5% CO$_2$ at 37°C. The resultant formazan crystals were dissolved using 100 μL isopropanol/0.04N HCl, absorbance was measured at $\lambda = 595$ nm using the BioTek ELx808™ microplate reader (BioTek Instruments, Inc, Winooski, VT, USA), and results were normalized to the control cells.

2.4 | Melanin content

MNT-1 melanotic melanoma cells were seeded in 6-well plates (Sarstedt, Nümbrecht, Germany) at the density of $0.3 \times 10^6$ cells/well, incubated with melatonin, its selected metabolites or serotonin in dose-dependent manner for 72 hours and assessed for melanin content. Cells were harvested, washed with 1 x PBS, centrifuged at 1,000 x g for 10 minutes at 4°C and incubated for 2 hours at 80°C in presence of 500 μL 1N NaOH. The absorbance was measured at $\lambda = 405$ nm using the BioTek ELx808™ microplate reader, and results were presented as the percentage of the control sample.

2.5 | Analysis of mitochondrial function

Melanoma cells were plated at the density of $0.25 \times 10^5$ cells/well and cultured on Seahorse XF-24 plates for 24 hours in a humidified atmosphere of 5% CO$_2$ at 37°C. Next day, cells were treated with tested compounds for 24 hours, all at the final concentration of $10^{-3}$ M versus control sample. Oxygen consumption rate (OCR or mitochondrial respiration) and extracellular acidification rate (ECAR or glycolytic function) in live cells in real time were measured using the Seahorse XF96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, USA) along the appropriately adapted procedure described previously.  Briefly, the initial 32 minutes reading established the baseline. Three subsequent injections followed, comprising oligomycin (complex V inhibitor), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, a proton gradient uncoupler that collapses proton electrochemical gradients and allows the respiratory chain to operate maximally) and rotenone/antimycin A, inhibitors for complex I complex III, respectively. Subsequent time points were recorded with approximately 30 minutes between each injection. For the ECAR assay, glucose, oligomycin and 2-deoxyglucose (2-DG) (a glucose analog, which inhibits glycolysis through competitive binding to glucose hexokinase, the first enzyme in the glycolytic pathway) were accordingly injected. Results for OCR and ECAR were automatically generated from Wave software data that has been exported to Excel and compared to the control sample.

2.6 | Evaluation of glucose consumption and oxidative stress

Glucose uptake and generation of reactive oxygen species (ROS) were analysed using colorimetric assays supplied by BioVision, Inc (Milpitas, CA, USA) where cells were seeded on 96-well plates ($0.15 \times 10^5$ cells/well), incubated with $10^{-3}$ M of melatonin, serotonin or metabolites for 24 hours and proceeded accordingly along the manufacturer’s instruction. Briefly, cells for glucose uptake were lysed with the extraction buffer for 40 minutes at 85°C, treated with the neutralization buffer and developed with the assay buffer. Assessment of generation reactive oxygen species was performed by H$_2$O$_2$ assay where reaction mix (Assay buffer, OxiRed™ Probe solution, HRP solution) was added to the cells, mixed thoroughly and incubated for 10 minutes at room temperature (RT). Differences were measured at $\lambda = 412$ nm and at $\lambda = 570$ nm for glucose uptake and ROS generation, respectively, using the BioTek ELx808™ microplate reader. Results were normalized to the control cells.

2.7 | Assessment of the mitochondrial membrane potential (mtΔΨ)

The detection of alterations within the inner electrochemical mtΔΨ in living cells was performed as described previously, using the cationic, lipophilic JC-1 dye (Thermo Fisher Scientific, Inc, Waltham, MA, USA). Briefly, cells were seeded in 6-well plates and allowed to attach overnight in a humidified atmosphere of 5% CO$_2$ at 37°C. The next day, cells were incubated with melatonin, its metabolites or serotonin at the dose of $10^{-5}$ or $10^{-3}$ M for 24 hours and then labelled for 15 minutes at 37°C with JC-1 (the final concentration: 1 μM in DMSO). Cells were centrifuged at 1,000 x g for 10 minutes at RT and resuspended in 500 μL 1 x PBS. The samples were kept on ice until analysis using
the FACSCalibur flow cytometer (Beckton Dickinson, San Jose, CA, USA). The mean of JC-1 fluorescence intensity was obtained from 10,000 cells using the excitation/emission settings at λex/em = 514 nm/529 nm. Results were analysed using the CellQuest Pro analysis software and expressed as a fluorescence ratio. Cells were additionally visualized by labelling using the JC-1 dye (Thermo Fisher Scientific, Inc, Waltham, MA, USA). Cells were seeded in an 8-well chamber slide (3.5 × 10^4 cells/well) and allowed to attach overnight in a humidified atmosphere of 5% CO_2 at 37°C. Next day, cells were stained for 15 minutes at 37°C with 1 µM JC-1 and incubated with melatonin, its metabolites or serotonin for 24 hours during which live imaging using the Olympus IX83 inverted Cell Vivo microscope (Waltham, MA, USA) was performed. During the whole experiment, cells were maintained under physiological conditions at 37°C and controlled humidity of 5% CO_2. Results were depicted as transition from JC-1 formed red-fluorescent “J- aggregates” to green-fluorescent JC-1 monomer.

### 2.8 | Transmission electron microscopy (TEM) assessment

Cells seeded in 6-well plates in supplemented MEM were treated with 10^{-3} M of melatonin, its metabolites or serotonin for 24 hours. Next, cells were harvested and centrifuged (700 r.p.m. for 5 minutes), washed with 1 × PBS, and fixation was performed for 24 hours at 4°C using 2.5% glutaraldehyde in 0.1 M cacodylate buffer. After that, cells were washed with 0.1 M cacodylate buffer, postfixed in 1% OsO_4 for 2 hours at RT and washed once more using distilled water. Cells were embedded in Poly/Bed®812 (Polysciences, Inc, Warrington, PA, USA) after dehydration in graded ethanol (50%-100%) and propylene oxide. Sections (65 nm thick) were stained with uranyl acetate/lead citrate prior to visualization using the Jeol JEM 2100 HT transmission electron microscope and assessed qualitatively in terms of the stages of melanosomes and shape of mitochondria.

### 2.9 | Statistical analysis

All the experiments were conducted at least (n = 4) and the results were expressed as the mean ± standard deviation (SD). Statistically significant differences between results were determined by the univariate analysis of variance (ANOVA) or the Student’s t test and appropriate post hoc analysis using GraphPad Prism 7.05 software (La Jolla, CA, USA). All the analyses are presented as percentage of the control sample, that is 0.2% EtOH, and a P-value of less than 0.05 was considered as statistically significant.

### 3 | RESULTS

#### 3.1 | Melatonin, its metabolites and serotonin affect cell proliferation and melanin content in melanoma cells

We compared melanotic (MNT-1) and amelanotic (A375, G361, Sk-Mel-28) melanoma cell lines whether melatonin, its precursor (serotonin) and its selected metabolites (both, from kynuric pathway (AFMK) and indolic pathway 6(OH)MEL, 5-MT) affected their viability. After 72 hours incubation, we observed significant drop of cell growth within the higher concentrations of all tested substances ranging from 10^{-5} M to 10^{-3} M and they appear to reveal similar pattern of regulation within all investigated melanoma cell models (Figure 1A–J). Namely, 10^{-3} M melatonin triggered collapse of proliferation ratio by 15% (G361, MNT-1), 19% (A375) and 23% (Sk-Mel-28) (Figure 1A and B). Comparatively, serotonin induced drop of viability by 13% (A375, G361, Sk-Mel-28) and 18% (MNT-1) (Figure 1C and D) while metabolites of melatonin-arrested melanoma growth ranging from 16% to 21% for AFMK, from 14% to 16% for 6(OH)MEL and from 15% to 33% for 5-MT (Figure 1E–J). Furthermore, a similar pattern of response was observed in terms of synthesis of melanin (Figure 2A–E). We noticed significantly (P < 0.001) lower melanin content versus control ranging from 14% to 21% for the dose of 10^{-5} M and from 15% to 54% for 10^{-3} M. The lowest concentration (10^{-9} M) led to proportionally smaller effect compared to intact cells ranging from 23% for serotonin, 16% for 5-MT to 12% for melatonin. Visualization of melanosomes in MNT-1 cells by transmission electron microscopy (TEM) and their qualitative assessment demonstrated that all the compounds at the dose of 10^{-3} M notably reduced the number of matured melanosomes (stage III-IV; yellow chevrons) with predominant accumulation of premelanosomes (stage I-II; white chevrons) (Figure 3A–F), and these responses are in line with one of our latest publications where melatonin significantly reduced melanogenesis.39

#### 3.2 | Melatonin, its precursor and its metabolites target mitochondrial function in melanoma cells

Numerous reports have shown that melatonin is present in cytosol, intracellular membranes as well as mitochondrial and nuclear compartments.68,69 This may suggest that melatonin-arrested cancer cell growth rate may be associated to their regulation/energy metabolism which is dependent either on mitochondria or the fact that cytosolic glycolysis for ATP synthesis is mostly used by cancer cells. Thus, two metabolic
pathways, OXPHOS or glycolysis, may be utilized as source of energy in metastatic melanomas depending on the actual environmental conditions. Herein, we assessed the impact of melatonin and its metabolites on mitochondrial respiration (OXPHOS)/glycolysis equilibrium quantified by real-time oxygen consumption rate (OCR) (Figure 4A–E) and extracellular acidification rate (ECAR or glycolytic function) (Figure 5A–D) in human MNT-1 melanoma cells. Firstly, assessment of OCR showed significant differences between control cells and mitochondrial parameters (Figure 4A–E and insert with respiration slopes). For instance, melatonin, its metabolites and serotonin significantly decreased basal respiration ranging

**Figure 1 (continues)**
from 11% to 15% (Figure 4A), enhanced twofold proton leak (Figure 4B), affected maximal respiration (Figure 4C), and as a result, spare respiratory capacity of melanoma cells was significantly attenuated (Figure 4D). These disturbances were in line with elevated oxidative stress. Thus, selected melanoma cells upon incubation with melatonin and its metabolites revealed significant elevation of reactive oxygen species (H$_2$O$_2$) versus intact cells ranging from 25% to 50% (Figure 4F and G). Besides, alterations within OXPHOS are accompanied by glycolysis disturbances in melanoma cells where similar
A pattern of regulation was noticed. Respiration slopes (Figure 5; insert) showed distinct ECAR differences between cells incubated with subjected substances and melanoma cells alone. Subsequent evaluation presented altered glycolysis itself (from 10% to 17% compared to the control cells) (Figure 5A). As a consequence, glycolytic capacity and glycolytic reserve were also affected (Figure 5B and C). These disturbances are in line with decreased glucose uptake in melanoma cell models reaching its drop ranging between 25%-35% for A375 and 23%-36% for G361 (Figure 5E) or between 24%-39% for MNT-1 and 28%-44% for Sk-Mel-28 (Figure 5F). Furthermore, comparative assessment of mitochondrial transmembrane potential (mtΔΨ) using selectively bound fluorescent dye (Figure 6A–C) was also performed. Live imaging of the changes within the inner mitochondrial membrane up to 24 hours showed a progressive dissipation of mtΔΨ in presence of melatonin and its metabolites. All compounds at 10⁻³ M caused significant increase of the green-fluorescent JC-1 monomer referred to decreased mtΔΨ. Thus, the ratio of red-to-green JC-1 fluorescence was dependent only on the membrane potential and not on other factors that may influence single-component fluorescence signals, such as size, shape and density of mitochondria. The results described above are in agreement with the visualization of mitochondria by transmission electron microscopy (see Figure 3A–F) where differences in morphology of mitochondria were observed as well.

4 | DISCUSSION

Mitochondria exert numerous functional responsibilities that make these organelles essential for cell survival. Among the intact cells, mitochondria are mainly responsible for ATP production occurring along the inner mitochondrial membrane. Glycolysis, taking place in the cytosol, generates pyruvate, which is subsequently transported into the mitochondrial matrix. Contrary to normal cells, mitochondria in cancer cells, including melanomas, exhibit markedly different metabolism. Thus, functionally altered mitochondria can jeopardize cellular homeostasis. For instance, “the mitochondrial diseases” such as Parkinsonism, cardiomyopathy, diabetes mellitus or cancer are all related to disturbed mitochondrial physiology. In addition, many other pathological conditions show dysfunctional mitochondria such as Alzheimer’s disease, intoxication with heavy metal, ischaemia/reperfusion injury, or exposure to ionizing and ultraviolet radiation. Herein, we assessed the impact of melatonin, AFMK, 6(OH)MEL, 5-MT and serotonin on mitochondrial functions in...
FIGURE 2 Melatonin and its metabolites decrease melanin content in human melanotic MNT-1 melanoma cells. Evaluation of melanin content after 72 h incubation with $10^{-9}$ M, $10^{-7}$ M, $10^{-5}$ M, $10^{-4}$ and $10^{-3}$ M melatonin (A), serotonin (B), AFMK (C), 6(OH)MEL (D) or 5-MT (E) as described in Materials and Methods. Data are presented as the mean ± SD (n = 6) and the results expressed as a percentage of the control cells (0.2% EtOH). Statistically significant differences versus control were indicated as *P < .05, **P < .01, ***P < .001 [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 3 Melatonin and its metabolites affect mitochondrial and melanosomal development in melanotic MNT-1 melanoma cells. Transmission electron microscopy images were obtained as described in Materials and Methods. This study reveals differences in the number and the shape of mitochondria as well as changes in particular stages of melanosomes 24 h after the treatment with melatonin (B), serotonin (C), AFMK (D), 6(OH)MEL (E) or 5-MT (F) at the dose of $10^{-3}$ M compared to the control cells (0.2% EtOH) (A). Mito: mitochondria; Nu: nucleus; Nucl: nucleolus; white chevrons: melanosome stage I–II; yellow chevrons: melanosome stage III–IV [Colour figure can be viewed at wileyonlinelibrary.com]
FIGURE 4 (continues)
various melanoma cell lines, one of the most complex, aggressive and heterogeneous cancers. Resistance to currently used anti-melanoma drugs and high safety profile of melatonin and its metabolites on the other side make an imperative to consider these substances into either novel therapies or complementary treatments of melanoma-affected patients. To date, it was shown that these compounds affect homeostasis of melanoma cell lines.\textsuperscript{10,39,75} Namely, they decrease proliferation rate what was previously shown in amelanotic rodent melanomas,\textsuperscript{41,76} and in human cutaneous cells.\textsuperscript{27,77} These inhibitory activities are in line with distinct number of studies showing reduction of cancer cell proliferation in lymphoid, prostate, carcinoma, and neuroblastoma.\textsuperscript{78-80} Differences in cancer cell sensitivity to melatonin or its metabolites may be connected to melanoma physiological imprinting of particular cell line. We used millimolar concentrations of compounds that in colon cancer cells decreased S-phase population and triggered apoptosis while the same concentration reduced the G2/M phase cells what was proved earlier in osteosarcoma or leukaemia cells.\textsuperscript{51} These differences are attributed to the general metabolic and differentiation states of cancer cells. Thus, the equilibrium between oxidative and glycolytic metabolism is regulated by hypoxia-inducible factor 1 (HIF-1), a transcription factor mediating the responses to changes in tissue oxygenation. Among the others, Slominski et al\textsuperscript{57} confirmed upregulation of the HIF-1 gene in the glycolytic pathway and increase of HIF-1 protein level during melanogenesis. Furthermore, it was shown that initiation of melanin synthesis is correlated with the expression of multiple genes involved in the regulation of melanocyte/melanoma behaviour, including the metabolic switch to glycolysis coordinated by HIF-1. These observations are in line with Park et al,\textsuperscript{82} Seagroves et al\textsuperscript{83} and Semenza\textsuperscript{84} who noticed significant decrease of melanogenesis at higher doses of melatonin. Since melatonin inhibits HIF-1α protein at such ranges, this indicates on the correlation between melatonin, HIF-1 and melanogenesis.

Contrary to listed above reports, we selected highly pigmented human melanotic MNT-1 melanoma cells in order to study the link between growth rate and melanin content what is in agreement with the previous publications in terms of pigmentation studies.\textsuperscript{59-61} In fact, melanoma cells originate from melanocytes which produce melanin in the skin under physiological conditions. Unlike normal melanocytes, melanoma cells do not excrete pigment to keratinocytes, leading to melanin accumulation and resultant heavy pigmentation of the cells. Melanin synthesis is deregulated in melanotic melanoma cells and this affects their behaviour but also of the surrounding environment with mutagenic immunosuppressive effects.\textsuperscript{56,57,85} As a result, this inhibits the host responses

\textbf{FIGURE 4} Melatonin and its metabolites alter oxidative phosphorylation in human melanoma cells. Seahorse mitochondrial respiration presents baseline, oligomycin-inhibited, FCCP-activated and rotenone/antimycin A-inhibited oxygen consumption rate (OCR) (insert). MNT-1 cells were treated for 24 h with $10^{-3}$ M of melatonin, its metabolites or serotonin and mitochondrial function was assessed (A–E). Comparatively, the appearance of $H_2O_2$ referred to generation of oxidative stress in amelanotic (A375, G361, Sk-Mel-28) and melanotic MNT-1 melanoma was investigated (F, G) as described in Materials and Methods. Data are presented as mean + SD (n = 6) and the results expressed as a percentage of the control cells (0.2% EtOH). Statistically significant differences \textit{versus} control were indicated as *$P<.05$, **$P<.01$, ***$P<.001$ for OCR assessment (A–E) and as #*$P<.05$, †$P<.01$, ‡$P<.001$ for oxidative stress (F, G).
and triggers the tumour progression. In our study, melatonin and its metabolites significantly reduced melanin content and this is consistent with the previous data. For instance, Kim et al. have shown that in humans, melatonin and some metabolites possess moderate inhibition of tyrosinase as well as affected proliferation of cultured epidermal melanocytes. In addition, it has been suggested that the cutaneous circadian clock regulates melanogenesis and melanocyte activities in human epidermis and hair follicles (HFs) with melatonin playing a role in this process. In this study, we operated
with the concentrations ranging from $10^{-9}$ M to $10^{-3}$ M while the mammalian plasma melatonin concentrations range of 10-200 pg/mL which corresponds to $5 \times 10^{-8}$ M to $10^{-6}$ M. On the other hand, Slominski & Pruski\textsuperscript{41} reported that at higher doses, melatonin possesses competitive inhibitory capacities more than acting via melatonin receptors. Melatonin at physiological doses ranging from 0.1 to 10 nM affects proliferation rate with no clear actions in terms of melanogenesis. Contrary, higher concentrations exceeding > 0.10 µM arrested melanogenesis without substantial impact on proliferation. Thus, different responses of melatonin on proliferation rate and melanin content may indicate that this substance regulates both processes through independent mechanisms. Besides, one of the most sensitive organelles for oxidative stress is mitochondria playing the key role of melanoma survival/death response, and which are the main target for melatonin. Thus, glucose regulation metabolism in cancer cells is essentially dependent on mitochondria. Herein, the assessment of the OXPHOS/glycolysis equilibrium showed on one hand reduction of oxidative phosphorylation with increased proton leak accompanied by enhanced oxidative stress while glycolytic capacity or glucose uptake were distinctly arrested on the other hand. These changes are in line with latest reports of Mi & Kuang\textsuperscript{86} or Puente-Moncada et al\textsuperscript{87} where glucose uptake significantly decreased by melatonin in HepG2 hepatic carcinoma or in tumour xenograft mouse model, respectively. Considering earlier reports, melatonin has been described as an effective anti-apoptotic, anti-inflammatory and anti-oxidative molecule in human keratinocytes or melanocytes under stress conditions.\textsuperscript{8,9,11-13,31} Herein, we noticed elevation of oxidative stress in melanoma cells which seems to be contradictory with reports mentioned above. It is postulated that the imbalance between cellular oxidative and anti-oxidant enzymes as well as overproduction of ROS in cancer cells have important treatments role.\textsuperscript{88} For instance, it was reported previously that melatonin therapy also generates ROS resulting in tumour cells.\textsuperscript{89} As a consequence, increased ROS due to melatonin leads to activation of apoptosis pathways such as caspase-3 in cancer cells.\textsuperscript{90} Furthermore, melatonin combined with endoplasmic reticulum stress affected antioxidant enzymes in melanoma cells.\textsuperscript{91} These observations are confirmatory of earlier mentioned statement that differences in cancer cell responses to melatonin and its endogenous metabolites may be related to their particular physiology compared to healthy cutaneous cells. This indicates that melatonin could be an appropriate candidate for melanoma treatment in combination with currently used therapies, however, further investigations should be carefully performed.
It should be also mentioned that melatonin is metabolized by cytochromes P450 (CYP450) in the liver, the key enzymes for endogenous drugs metabolism.\(^9\) It was shown previously that melatonin is metabolized to 6(OH)MEL and N-acetylserotonin (NAS) by CYP1A1, and most of them are converted to sulphate derivatives in human liver.\(^9\) CYP1B1 is engaged in melatonin metabolism to NAS in mitochondria of the tumour cells. Since melatonin crosses the cell membrane and penetrates many cell compartments, its facilitated diffusion in transmembrane transportation was recently reported.\(^9,12,27,31,32,37,39\) For instance, it is known that oligopeptide transporter 1/2 (PEPT1/2) renders its oncostatic activity in tumour cells and it was described to facilitate melatonin transportation into mitochondria. As a matter of fact, its accumulation in these organelles triggered apoptosis of human cancer cell lines.\(^94\) Glycolysis and OXPHOS are two energy-producing metabolic pathways in melanoma cells and most of cancer cells exert the ability to switch between these two mechanisms allowing to adapt to environmental changes.\(^70,95\) Thus, we showed within this study that melatonin as well its metabolites, what is brand new in case of AFMK, 6(OH)MEL, 5-MT and serotonin, suppress proliferation ratio, decrease melanogenesis and reduce OXPHOS/glycolysis equilibrium or glucose homeostasis.

In conclusion, enclosed data within this study together with the previous reports prompt us to postulate that incorporation of these substances into routine medical therapies, including surgery, chemotherapy, radiotherapy and modern targeted therapy may reduce side effects and enhance survival rate of melanoma-affected patients. Thus, melatonin, its precursor (serotonin) and its metabolites affect melanoma proliferation, melanin content and their mitochondrial functions (Figure 7). These results suggest to consider their use either alone or in combination with currently used anti-melanoma agents in patients for whom immunotherapy fails. Nevertheless, it is important to test these associations on melanoma patients in clinical setting.
FIGURE 6  Melatonin and its metabolites decrease mitochondrial transmembrane potential (mtΔΨ) in human melanoma cells. MNT-1 cells were treated with melatonin, serotonin, AFMK, 6(OH) MEL or 5-MT and evaluated with regard to changes within mitochondrial function by fluorescence labelling using the JC-1 dye (A–C) as described in Materials and Methods and assessed by live imaging for 24 h (B). Comparative flow cytometry analysis was performed (C) subsequently quantified and results presented as mean ± SD (n = 4). The values are expressed as a percentage of the control cells (0.2% EtOH). Statistically significant differences versus control were indicated as *P < .05, **P < .01, ***P < .001. Bar = 50 μm
CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION
KK designed the concept of the studies, conducted the progress of analysis, together with BB and FS analysed all the results, and wrote the first draft of the manuscript. BB completed electron microscopy quantification and together with EP described these data. MKT carried out mitochondrial assessment and together with KK evaluated obtained results. AP and MZ carried out fluorescence imaging and described the results. JS together with KK evaluated glucose uptake and H2O2 assays. KK, BB and FS interpreted collectively pooled data, subsequently together with AP, JS, EP, MZ, RJR, KS, ATS and MKT drafted and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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