



Photodynamic therapy-induced killing is enhanced in depigmented metastatic melanoma cells

Krishna V. Sharma, Natalie Bowers and Lester M. Davids¹

Redox Laboratory, Department of Human Biology, Faculty of Health Sciences, University of Cape Town, Observatory, 7925 Cape Town, South Africa

Abstract

The resistance of pigmented human melanomas over their unpigmented counterparts to a number of therapies has suggested that the presence of intracellular melanin plays a role in rendering these cells less susceptible to cell death, probably through the ability of this pigment to act as an intracellular antioxidant, thus neutralizing chemotherapeutic-induced ROS (reactive oxygen species). PDT (photodynamic therapy) was recently suggested as an attractive, adjunctive therapy owing to its cellular specificity and limited side effects. In the present study, we propose that first depigmenting melanomas with a reversible TYR (tyrosinase) inhibitor such as PTU (phenylthiourea) increases their susceptibility to HYP-PDT (hypericin-mediated PDT). Pigmented [UCT Mel-1 (University of Cape Town melanoma cell line 1)] and unpigmented (A375) melanomas were first characterized with respect to their TYR activities and melanin quantities and then treated with a TYR inhibitor for 48 h. Cell viability assays after treatment with 3 μ M HYP-PDT showed a significant increase in cell death in depigmented melanomas compared with untreated melanomas that returned to the level of untreated melanoma cells on removing the TYR inhibitor. The present study supports the hypothesis that combining the inhibition of melanogenesis with PDT should be explored as a valid therapeutic target for the management of advanced melanoma.

Keywords: hypericin; melanin; melanoma; phenylthiourea; photodynamic therapy; tyrosinase inhibition

1. Introduction

Despite the extensive research and clinical trials so far, the prognosis and survival of metastatic melanoma remain dismal. Because melanoma is inherently resistant to traditional forms of adjuvant therapy such as chemotherapy and radiotherapy (Zbytek et al., 2008), various other strategies have been developed including immunotherapy using IL-2 (interleukin-2) (Carlson et al., 2005), radiotherapy (Moncrieff et al., 2008), biochemotherapy (Slominski et al., 2001, 2004; Chudnovsky et al., 2005; Yamamura et al., 2008) and gene therapy (Carlson et al., 2005; Thompson et al., 2005). Although a limited number of these therapies have progressed to human clinical trials, their outcomes remain negligible.

The need for alternative therapeutic intervention is thus supported. One such alternative is PDT (photodynamic therapy). PDT is a minimally invasive therapeutic modality that has been shown to be effective in several types of cancer and non-oncological conditions (Buytaert et al., 2007). The basis of PDT is the systemic or topical application and preferential uptake of a PS (photosensitizer) by the tumorigenic cells, which upon light activation produces ROS (reactive oxygen species). These in turn produce a cascade of molecular and biochemical events resulting in cell death via apoptotic or necrotic mechanisms (Henderson and Dougherty, 1992; Dougherty et al., 1998).

Hypericin is a relatively new, second-generation PS with an enhanced stability and a rapid, efficient mechanism of action. Owing to its hydrophobic nature, it passively crosses all cell membranes, and activation can occur at two absorption peaks

(Milanesio et al., 2001; Dolmans et al., 2003). As a follow-on of earlier studies using HYP-PDT (hypericin-mediated photodynamic therapy) to combat melanomas, we have shown that even though HYP-PDT at a concentration of 3 μ M reduces cell viability in melanoma cells, there is a clear differential susceptibility between pigmented and unpigmented melanoma cells with pigmented cells being less susceptible to PDT killing (Davids et al., 2009). A number of recent studies have suggested that the intractability and inherent refractiveness of melanoma to chemotherapeutics and other therapy modalities are perhaps related to the presence of the polymeric pigment melanin within melanomas (Wood et al., 1999; Farmer et al., 2003; Chen et al., 2009, 2006), the present study hypothesizes that depigmenting melanoma cells increases their susceptibility to adjuvant PDT treatment, leading to a significantly enhanced susceptibility to cell death.

This hypothesis was further supported by earlier work of Hadjur et al. (1996), who showed that HYP-PDT phototoxicity in pigmented melanoma is lower than that in unpigmented melanoma owing to the suggested photoprotective properties of melanin to ROS (Hadjur et al., 1996). In addition, Slominski et al. (1998) showed that melanoma cells with differing melanin contents show increased susceptibility to ionizing radiation (Slominski et al., 1998) and this same group recently showed that suppression of melanogenesis increases the effectiveness of chemotherapeutic agents (Brozyna et al., 2008; Slominski et al., 2009). In the present study, we therefore tested the effects of suppression of melanogenesis using a known TYR (tyrosinase) inhibitor, PTU (phenylthiourea), on characterized pigmented melanoma cells, followed by HYP-PDT. We

¹To whom correspondence should be addressed (email lester.davids@uct.ac.za).

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; dopa, 3,4-dihydroxyphenylalanine; FBS, fetal bovine serum; H&E, haematoxylin and eosin; HYP-PDT, hypericin-mediated photodynamic therapy; PDT, photodynamic therapy; PS, photosensitizer; PTU, phenylthiourea; ROS, reactive oxygen species; TYR, tyrosinase; UCT Mel-1, University of Cape Town melanoma cell line 1.

show that depigmented cells display an enhanced death susceptibility to HYP-PDT which approaches that of unpigmented melanoma cells. This further strengthens the hypothesis that in melanomas, melanin acts as a 'two-edged' sword – on the one hand protecting cells against ROS and on the other hand acting as a potent cytotoxic compound on PDT-induced leakage from the melanosomes – and that this must be considered in future strategies to combat melanoma.

2. Materials

All chemicals were purchased from Sigma–Aldrich unless otherwise stated. Phenylthiourea was prepared as a 150 mM stock in 100% ethanol and further dilutions were made in distilled H₂O. Melanin was prepared as a 3 mg/ml stock in sodium hydroxide and hypericin was prepared as a 2 mM stock in DMSO (BDH Merck). DMEM (Dulbecco's modified Eagle's medium) and FBS (fetal bovine serum) were obtained from Highveld Biological.

3. Methods

3.1. Cell cultures

The human pigmented melanoma cell line [UCT Mel-1 (University of Cape Town melanoma cell line 1), metastatic nodal] was derived from excised tissue obtained from consenting melanoma patients at Groote Schuur Hospital (Cape Town, South Africa). The unpigmented melanoma cell line (A375, primary cutaneous) was purchased from the A.T.C.C. All melanoma cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% penicillin (100 units/ml) and streptomycin (100 µg/ml). The cells were incubated in 5% CO₂/95% air at 37°C in a humidified incubator. Both cell lines were routinely checked for mycoplasma contamination and only the cells that were clear of any contamination were used in further experimental procedures.

3.2. Melanin content determination

Melanin content was determined by using a well-established protocol (Siegrist and Eberle, 1986). Briefly, 1×10^6 cells/ml were pelleted and resuspended in 1 M NaOH and solubilized on a heating block at 100°C for 30 min. The cells were centrifuged at 16000 g for 20 min at room temperature and the supernatant was removed and read in duplicate in a 96-well plate in aliquots of 200 µl. The absorbance was read at 400 nm on a spectrophotometer (VERSAmax tunable microplate reader; Labotec Molecular) and compared with a standard curve prepared with synthetic melanin standards ranging from 0 to 300 µg/ml (melanin from *Sepia officinalis*; Sigma).

3.3. TYR activity assay

TYR assays were performed with protein extracted from melanoma cells by using an established protocol (Virador et al., 1999).

Briefly, this involved adding 30 µl of cell extract to a 96-well microtitre plate in duplicate, along with 10 µl of L-[¹⁴C]tyrosine (50 µCi/mmol; AEC Amersham) and 10 µl of 0.25 mM dopa (3,4-dihydroxyphenylalanine) cofactor in 0.1 M sodium phosphate buffer (pH 7.2). The samples were incubated in a 37°C incubator for 1 h, after which it was placed on ice and left to cool for 5 min. Then, 40 µl of the reaction mixture was spotted on to Whatman glass microfibre filters and allowed to dry for 15 min at room temperature; then it was subjected to a series of hydrochloric acid washes, followed by a 95% ethanol (Merck) wash and a 5 min wash in acetone (Merck). The filters were placed in 5 ml of scintillation fluid (Amersham) and read on a scintillation counter (Beckman). TYR activity is reported as c.p.m./µg of protein per h.

3.4. The dopa oxidase assay

The dopa oxidase reaction visualizes the presence of melanin in cells due to the addition of dopa, the substrate for the enzyme TYR. It is thus a visual confirmation of TYR activity and melanin content in cells. Briefly, 5×10^4 cells are seeded on to a glass coverslip and allowed to adhere overnight at 37°C. A 0.1 M dopa (Sigma)/0.1% Triton X solution (in phosphate buffer, pH 7.2) was added to the cells for 1 h. Cells were then fixed in ice-cold methanol for 30 min, counterstained with H&E (haematoxylin and eosin) dye for 1 min and then mounted on glass slides. Slides were viewed under a brightfield filter on a Zeiss Axiophot microscope with associated software.

3.5. PTU treatment

A concentration range of 0–300 µM PTU was made in PBS (pH 7.2) and ethanol (final concentration <0.1%) at room temperature and added to the melanoma cells in culture to determine a cytotoxicity profile. The optimal concentration of PTU was then added for a period of 48 h prior to hypericin treatment. Controls included were cells incubated with PTU vehicle (0.1% ethanol) only.

3.6. Hypericin treatment and UV irradiation

Concentrations of hypericin and UV irradiations followed a previously established protocol (Davids et al., 2008).

3.7. Cell viability assay

The Cell Titer proliferation assay (Roche) was used to evaluate cell viability after PTU-mediated depigmentation and hypericin-UVA treatment. Briefly, cells were seeded in a 96-well microtitre plate at a density of 1.5×10^4 per well and left at 37°C overnight. Cells were then incubated with PTU for 48 h, washed with PBS and then incubated with hypericin for 4 h. Thereafter, the hypericin was activated with 1 J/cm² of UV-A (320–400 nm, maximum spectral output at 365 nm) and the cells were allowed to recover in culture medium for 24 h. At 4 h before the end of the recovery period, XTT (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide) solution was added for 4 h at 37°C and the plate was read at 450 nm on a spectrophotometer (VERSAmax tunable microplate reader; Labotec Molecular) with associated

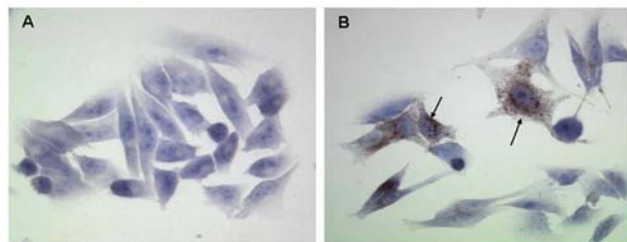


Figure 1 Dopa oxidase assay showing the (A) absence and (B) presence of melanin granules within melanosomes of the unpigmented A375 and pigmented UCT Mel-1 melanoma cell lines respectively

Black arrows indicate the peri-nuclear distribution of melanin granules in the pigmented cells. All pictures were taken at $\times 400$ magnification and counterstained with H&E.

SOFTmaxPRO 4.3.1 software. Attenuance results are presented as a percentage of the untreated control values. Results are reported as means \pm S.E.M. for four independent experiments.

3.8. Statistics

Statistical analyses were conducted using the paired Student's *t* test for significance with 95% CI (confidence interval) ($P < 0.05$). All graphs and statistics were prepared using the GraphPad Prism software package.

4. Results

4.1. Melanoma characterization

In order to test the effect of hypericin on PTU-mediated depigmented melanomas, it was imperative to characterize the melanoma cell lines with respect to their pigmentation. Pigmentation was assessed in a number of ways. It was visually assessed through the intracellular dopa oxidase assay (see section 3). As can be seen in Figure 1(A), the unpigmented melanoma cells (A375) lacked functional dopa oxidase activity from the rate-limiting enzyme TYR and hence displayed cells devoid of pigmented melanosomes (Figure 1A). Interestingly, electron microscopic analyses of these cells show the presence of structural 'empty' melanosomes containing no melanin (Davids et al., 2009). In contrast, the pigmented melanoma cell line, UCT-Mel-1, displayed typical peri-nuclear-located dopa oxidase-positive, pigmented melanosomes (Figure 1B, arrows). Pigmentation was also measured using the TYR and melanin assay to measure total TYR activity and associated melanin content respectively. As expected, the TYR activities for both the pigmented (Figure 2A, black bar) and the unpigmented (Figure 2A, grey bar) melanoma cells not only correlated with the melanin content for both the pigmented and unpigmented melanoma cell lines but also showed significant difference between the two cell lines (Figures 2A and 2B). In addition, cell pellets visually confirmed the presence and absence of pigment correlated to the cell lines (Figure 2B, inset). The UCT Mel-1 pigmentation status correlated to the commercially available pigmented metastatic melanoma cell line, 501, which we had previously used (results not shown). As such, these cells were

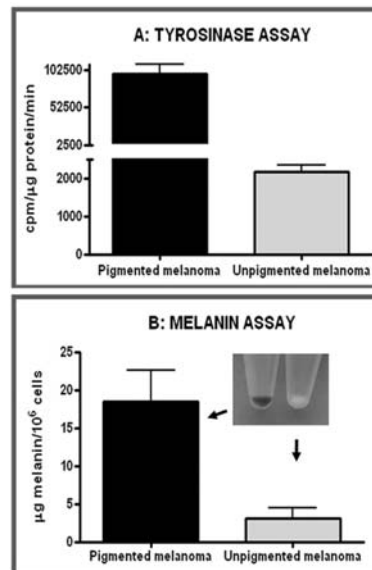


Figure 2 Phenotypic characterization of the melanoma cell lines with respect to (A) total TYR enzyme activity and (B) melanin quantification

Graphs were created from at least three independent experiments and results shown are means \pm S.E.M.

therefore considered characterized with respect to their melanogenic status and could be treated with the TYR inhibitor.

4.2. Effect of PTU on depigmentation

PTU displayed a dose- and time-dependent decrease in TYR activity (Figure 3) and melanin content (Figure 4) over both 2 and 5 day exposure periods in the pigmented melanoma cell line, UCT Mel-1. Specifically, 300 μ M PTU resulted in TYR activities of $42.84 \pm 1.67\%$ and $66.90 \pm 0.65\%$ after 2 and 5 days respectively (Figure 3). In contrast, 300 μ M PTU only led to a slight decrease in melanin formation over the same time periods ($92.14 \pm 4.69\%$ and $75.32 \pm 15.79\%$ total melanin for 2 and 5 days respectively; Figure 4). Moreover, it shows that with even low TYR activity after 2 days of treatment, the amount of melanin generated is not significantly lowered. These inhibition data corroborate the work of Ni-Komatsu et al. (2005), who showed that 300 μ M in melanocytes produces effective TYR inhibition over a 24 h period. However, their study did not expose cells for longer than 24 h (Ni-Komatsu et al., 2005). No cytotoxicity was observed in the pigmented melanoma cell line at any of the concentrations tested after 24 h (Supplementary Figure S1 available at <http://www.cellbiolint.org/cbi/035/cbi0350939add.htm>). These results highlight the specificity of the PTU as a TYR activity inhibitor but the increase in TYR activity after 5 days also shows that this treatment is reversible. On the basis of this, we chose to pretreat the melanomas for 2 days only before the HYP-PDT experiments.

4.3. Effect of PTU-HYP-PDT treatment on melanoma cells

To test the effect of HYP-PDT on PTU-mediated depigmentation in the pigmented melanoma cell line, both 1 and 3 μ M hypericin

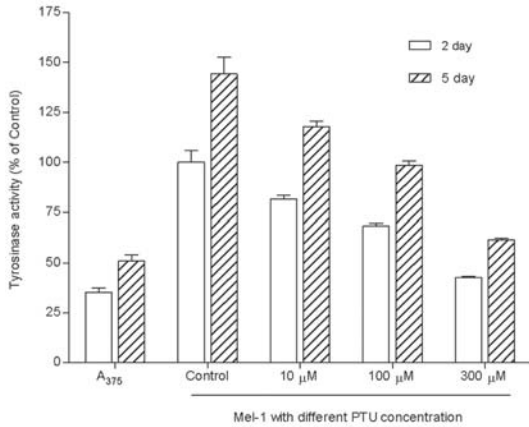


Figure 3 The effect of PTU treatment on the TYR activity of the pigmented melanoma cell line, UCT Mel-1, over a period of 2 and 5 days

Graphs were created from at least three independent experiments and results are plotted as mean percentage of the control \pm S.E.M. Controls represent untreated cells.

was used to treat the cells after 2 days of depigmentation with 300 μ M PTU. We have previously shown in these cell types that 1 μ M is a viable hypericin concentration upon activation with 1 J/cm² UVA light, whereas 3 μ M hypericin exposure is cytotoxic (Davids et al., 2008). A non-melanoma skin cell type (keratinocytes) was included as a negative control. Figure 5 shows that activated 1 μ M hypericin had no significant effect on the cell viability of any of the cell types. In contrast, exposure to 3 μ M HYP-PDT significantly decreased the viability of pigmented UCT-Mel 1 melanoma cells to 55.69 \pm 6.76% (Figure 5, black bar). Significantly ($P < 0.05$), HYP-PDT treatment on pigmented melanoma cells that had been depigmented for 2 days with PTU led to a 3.5-fold decrease in cell viability compared with the unpigmented treated cells (55.69 \pm 6.76% versus 15.60 \pm 2.84%, Figure 5, grey, solid bar) which was comparable with the unpigmented A375 melanoma cells (18.18 \pm 4.11%, Figure 5, chequered bar). No difference in cell viability was detected in the keratinocyte skin cells at both hypericin concentrations (Figure 5, white bar).

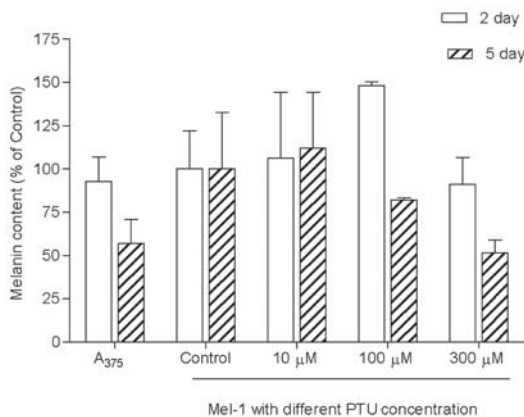


Figure 4 The effect of PTU treatment on the melanin quantity of the pigmented melanoma cell line, UCT Mel-1, over a period of 2 and 5 days

Graphs were created from at least three independent experiments and results are plotted as mean percentage of the control \pm S.E.M. Controls represent untreated cells.

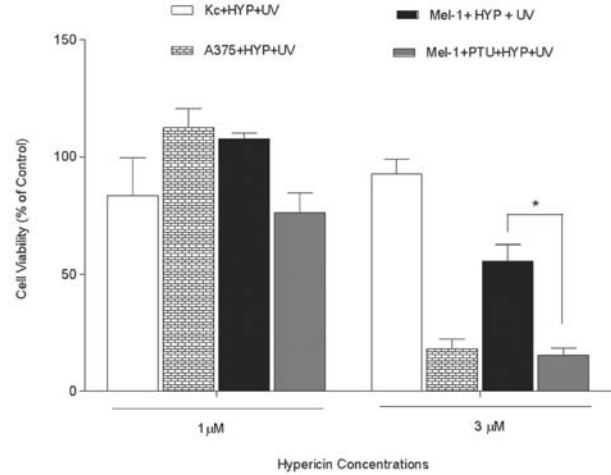


Figure 5 A graph representing the effect of HYP-PDT after 2 days treatment with 300 μ M PTU

Results are presented as percentages of cell viability compared with untreated control (0 μ M hypericin) and represent mean percentages \pm S.E.M. for three independent experiments. Significance was calculated using a paired Student's *t* test ($*P < 0.05$).

The PTU treatment was then removed and the cells allowed to fully recover for a period of 2 days during which time the TYR activities and melanin content returned to normal levels (results not shown). At this point, they were again exposed to HYP-PDT. Figure 6 shows no significant difference between any of the cell types and their controls after exposure to 1 μ M hypericin. Despite an expected overall decrease in cell viability due to 3 μ M HYP-PDT in all the melanoma cells, previously treated PTU melanomas (39.23 \pm 9.56%, Figure 6, grey bar) displayed no significant decrease in cell viability compared with untreated cells (47.94 \pm 13.33%, Figure 6, black bar) or unpigmented A375 melanoma cells (56.00 \pm 15.55%, Figure 6, chequered bar), showing that pretreatment with PTU causes an enhanced susceptibility in pigmented melanomas to PDT. The keratinocytes once again showed no difference in cell viability between the

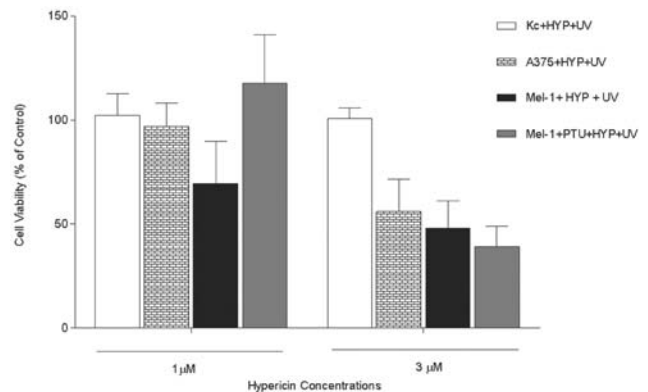


Figure 6 A graph representing the effect of HYP-PDT on the pigmented melanoma cells after removing the TYR inhibitor for 2 days

Results are presented as percentages of cell viability compared with untreated control (0 μ M hypericin) and represent mean percentages \pm S.E.M. for three independent experiments.

lower and higher exposed concentrations of hypericin (Figure 6, white bar).

5. Discussion

Melanoma continues to be one of the most difficult cancers to treat. Apart from its high proliferative index, invasiveness and metastatic potential, it has been shown to be inherently refractory to chemotherapeutic treatments (Slominski et al., 1998; Wood et al., 1999; Chen et al., 2009). Part of this intractability has been suggested to be related to the presence of the polymeric pigment, melanin. Melanin, a light-absorbing biopolymer with electromagnetic properties, is exclusively produced in cells of the melanocytic lineage and has been shown to not only scavenge free radicals and ROS but also chelate chemotherapeutic agents and produce relatively hypoxic environments due to increased oxygen consumption (Wood et al., 1999; Slominski et al., 2004). The formation of melanin is via the melanogenic pathway that comprises a series of catalytic reactions driven by the rate-limiting enzyme TYR. TYR, the rate-limiting enzyme of the pathway, and its related proteins TYR1 and TYR2 (TYR-related proteins 1 and 2) act in concert to first convert tyrosine to dopa via tyrosine hydroxylase activity and then convert dopa to dopaquinone via dopa oxidase activity. Both of these activities occur via separate TYR catalytic sites. During melanin synthesis, toxic intermediates such as 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid are produced. Structurally, the melanosomes are designed to compartmentalize these cytotoxic melanin intermediates from spilling into the cytoplasm. Melanosomal biogenesis progresses through four distinct stages of maturation where the first two stages contain no melanin and the later stages constitute intermediates required in order to generate a matrix favourable for the formation of melanin (Solano et al., 2000; Raposo and Marks, 2002).

Brozyna et al. (2008) in a series of elegant experiments showed that melanin acts as a 'double-edged' sword in the cell – on the one hand as an oxidant if leaking from the melanosomes and on the other hand as an antioxidant through its ability to quench ROS produced via chemotherapeutic drugs (Brozyna et al., 2008). On the basis of our previous results (Davids et al., 2008, 2009), our hypothesis for the present study proposed therefore that the presence of melanin in HYP-PDT-treated cells acts as an antioxidant, neutralizing the efficacy of PDT treatment. With this in mind, we tested the effects of suppression of melanin formation (melanogenesis) on the effectiveness of killing melanoma cells by HYP-PDT treatment. We used two human melanoma cell lines – one pigmented (UCT Mel-1) and unpigmented (A375) – which we fully characterized with respect to their phenotypes and then pretreated them with a well-known TYR inhibitor (48 h), PTU, followed by HYP-PDT treatment and measured their susceptibility to cell death. PTU has long been known as a reference inhibitor (DuBois and Erway, 1946) against TYR (Criton et al., 2008). In addition, it has been shown to inhibit the catechol oxidase enzyme that belongs along with TYR to the type-3 copper proteins group. Its mechanism of action on TYR relates to the sulfur atom of the

PTU binding to both copper ions in the active site to block enzyme activity (Klabunde et al., 1998; Gerdemann et al., 2002).

The data clearly show that the melanotic phenotype attenuates PTU+HYP-PDT toxicity and, conversely, that inhibition by a known TYR inhibitor sensitises/increases the susceptibility of these melanoma cells to HYP-PDT. In addition, on removing the TYR inhibitor and thus allowing for fully functional TYR activity and melanin formation, the pigmented cells showed an increased resistance to PDT-induced cell death. These results are consistent with the known chemical properties of melanin being a strong detoxifying agent and ROS scavenger (Wood et al., 1999; Hoogduijn et al., 2004, 2003) and reiterate that in a clinical setting, the concentration and frequency of administration of TYR inhibition would have to be optimized. Moreover, the induced increased susceptibility of depigmented melanoma cells to HYP-PDT suggests a new adjunctive therapeutic avenue for the control of this intractable disease in advanced stages. Thus these findings, in conjunction with the recently reported radiosensitization of melanotic melanoma cells by the inhibition of TYR activity (Brozyna et al., 2008), support the need for further efforts for the development of novel therapies for melanotic melanoma based on inhibition of melanogenesis. Undoubtedly, future work would have to consider *in vivo* assays to assess the clinical efficiency of this treatment as well as potential side effects. However, the results of the present study contribute to using the inhibition of melanogenesis to represent the dawn of novel adjuvant therapeutic modalities.

In summary, we provide further evidence in support of the hypothesis that inhibition of melanogenesis should be explored as a valid adjunctive therapy towards combating metastatic melanoma.

Author contribution

Lester M. Davids was the group leader and designer of research and experiments and wrote most of this paper. Krishna V. Sharma completed most of the experimental work and participated in the writing of the manuscript. Natalie Bowers contributed by conducting the initial experiments.

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