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Melanin as a Target for Melanoma Chemotherapy: Pro-oxidant Effect of Oxygen and Metals on Melanoma Viability

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Melanoma cells have a poor ability to mediate oxidative stress, which may be attributed to constitutive abnormalities in their melanosomes. We hypothesize that disorganization of the melanosomes will allow chemical targeting of the melanin within. Chemical studies show that under oxidative conditions, synthetic melanins demonstrate increased metal affinity and a susceptibility to redox cycling with oxygen to form reactive oxygen species. The electron paramagnetic resonance (EPR)-active 5,5′-dimethyl-pyrollidine N-oxide spin adduct was used to show that binding of divalent Zn or Cu to melanin induces a pro-oxidant response under oxygen, generating superoxide and hydroxyl radicals. A similar pro-oxidant behaviour is seen in melanoma cell lines under external peroxide stress. Melanoma cultures grown under 95% O_2/5% CO_2 atmospheres show markedly reduced viability as compared with normal melanocytes. Cu- and Zn-dithiocarbamate complexes, which induce passive uptake of the metal ions into cells, show significant antimelanoma activity. The antimelanoma effect of metal- and oxygen-induced stress appears additive rather than synergistic; both treatments are shown to be significantly less toxic to melanocytes.

Key words: Pro-oxidant, Melanins, Melanoma, Oxygen, Metal uptake, Melanocytes

INTRODUCTION

Melanomas are among the most drug resistant cancers, and even with chemotherapy the prognosis for disseminated melanoma is poor (1). Tyrosinase, the melanin-synthesizing enzyme unique to melanogenic cells, has been proposed as a target for chemotherapy (2), efforts on this approach are still underway (3). A characteristic of melanomas is abnormal, poorly compartmentalized melanosomes, with malformed or twinned membranes, occlusions within the melanin, and fragments evident outside of melanosome (4, 5). These structural differences are significant, as melanosomal compartmentalization helps protect the cell from the reactive catechols that are generated during melanogenesis. Such protection is likely abbreviated or lost within melanomas; we propose that the complex reactivity of these melanoid fragments may provide a unique target for chemotherapy.

Several recent studies suggest that melanomas are predisposed to oxidative stress (6, 7). Large differences between the ability of melanocytes and melanoma cells to mediate oxidative stress have been demonstrated in studies of cell lines exposed to extra-cellular stress (8, 9). Melanomas also have unusually high uptake of Cu and other metals, even higher than melanocytes which themselves accumulate metal ions (10). More importantly, melanomas have been shown to be uniquely susceptible to increases of certain divalent metal salts; for instance, Zn(II) and Cd(II) salts induce melanoma death at concentrations several orders of magnitude lower than that which affects melanocytes (11, 12).

Melanin particles have a high affinity for metal ions in vivo (13), and binding of metal ions dramatically affects certain chemical properties of the melanin (14, 15). For example, Zn(II) binding shifts the melanin-based oxidation potential by

Abbreviations – CL, chemiluminescence; DHI, dihydroxyindole; DMPO, 5,5′-dimethyl-pyrollidine N-oxide; DTC, dithiocarbamate; ROS, reactive oxygen species

c. 100 mV, making it more easily oxidized, and Cu(II) binding dramatically increases the rate of melanin bleaching (16). We have found evidence that the initial oxidation of melanin produces a quinone-imine tautomer, whose concentration tracks with metal-binding and chemical degradation (14). The quinone-imine has a high affinity for metal ions, with an effective $K_a > 10^{11}$ for Cu(II) at pH 7 (14). Thus melanin's metal-binding ability increases upon partial oxidation, which may increase its susceptibility to further oxidation.

The interaction of melanins with oxygen has been extensively studied by Sarna and colleagues (17, 18). Peroxide is the main product of the bleaching of melanin in air (17), and the rate of this bleaching may be increased by the presence of certain metal ions (18). In this work, we examine the prooxidant effect of divalent Cu and Zn on synthetic melanins, in comparison with the response observed for melanoma cells under peroxide stress. The resulting implications are then tested in viability studies of melanoma and melanocyte cell cultures under oxygen and Cu and Zn-induced stress.

MATERIALS AND METHODS

General

5,6-Diacetoxyindole (DAI) was obtained from TCI America (Portland, OR, USA). 5,5-dimethyl-1-pyroline-N-oxide (DMPO), superoxide dismutase (from bovine erythrocyte), catalase (from mouse liver) and agarose gel were purchased from Sigma (St Louis, MO, USA). *Escherichia coli* and *pUC-18* plasmid DNA were purchased from Stratagene (La Jolla, CA, USA). Indium tin oxide plates were purchased from Quantum Coating (Mt Laurel, NJ, USA). Pyrolytic graphite (PG) was purchased from Advanced Ceramics (Cleveland, OH, USA). Plasmid Midi Kit was from QIAGEN Inc. (Valencia, CA, USA). DNA Clean and Concentrator^{TM}-5 kit was purchased form Zymo Research (Orange, CA, USA). A Barnstead nanopure system was used to purify water to a specific resistance of 18 M/cm. All other chemicals were reagent grade quality.

DHI-melanin Studies

Electrochemical experiments were carried out with a BAS-100B-W electrochemical analyser. A three-electrode voltammetric cell was used with a platinum counter electrode, Ag/AgCl reference electrode, and hand cut ITO or PG plates as working electrode. All reported potentials are vs. Ag/AgCl reference electrode.

Poly-dihydroxyindole (DHI) films were prepared by bulk electrolysis of DHI solution in a glove-box under a nitrogen atmosphere, as described in (14). A DHI solution was generated by hydrolysis of DAI in situ. Polymerization of DHI on electrode surface was achieved using bulk electrolysis at 20 mV vs. Ag/AgCl. The resulting films were then rinsed several times with deionized water and stored in the glove-box. Cu and Zn treated poly DHI films were prepared by soaking the premade films in a 0.1-M $CuCl_2$, or 0.1 M $Zn(CH_3COO)_2$ solution under nitrogen atmosphere overnight. The poly DHI films were then rinsed with deionized water several times, dried and stored under nitrogen.

DMPO Trapping

Electron paramagnetic resonance (EPR) experiments were run on a X-band 300 EMX spectrometer (Bruker BioSpin Corp. Billerica, MA, USA) equipped with a high sensitivity cylindrical cavity (ER 4103TM). Measurements were carried out using a 0.3-mm flat cell. Poly DHI films on PG substrates were taken out of the glove-box and immediately placed in a *c*. 500 µl DMPO solution (70 mM in 100 mM pH 7.0 phosphate buffer) in the dark. There was a large molar excess (*c*. 200–1) of DMPO in solution in comparison with the DHI subunits electrodeposited on the surface of the electrode. In experiments with catalase and superoxide dismutase, the DMPO solution contained 40 µg/ml catalase or 20 µg/ml superoxide dismutase. After 2 h, the poly DHI films were removed and the solutions were analysed by EPR. The DMPO-OH signal intensities were normalized to the relative amount of poly DHI in each film by dividing the signal height by the charge passed during the electrodeposition, at a variance of $< 5\%$ within any set of experiments.

Hydroxyl Assay

To prepare *pUC-18* plasmid DNA, the plasmid was introduced to *Escherichia coli* cells by PEG-DMSO transformation method (20). The cells were plated out on to nutrient LB agar medium containing ampicillin. Plasmid isolation and detection were carried out according to QIAGEN plasmid purification handbook using QIAGEN Plasmid Midi Kit. The purified DNA was diluted in 100 mM pH 7.0 phosphate buffer to a final concentration of 30 ng/µl. Poly DHI films on PG were placed in aerated DNA solution for 2 or 24 h. The films were then removed and DNA solutions were concentrated using DNA clean & concentrator^{TM}-5 kit from Zymo Research. Gel electrophoresis of plasmid DNA was carried out in 1% molecular biology grade agarose gel containing ethidium bromide at 110 V for 45 min. The bands were illuminated with UV light and photographed.

Cell Culture Studies

Metastatic melanoma cell strains (c81-46A, c83-2C, c81-61 and A375) were cultured in F-10 medium (Fisher Scientific, Pittsburgh, PA, USA) containing cysteine (25 mg/l) and methionine (4.5 mg/l) with additions of 5% foetal calf serum, 5% newborn calf serum. The passage number used in these experiments was not higher than 10. All cell strains grow in a monolayer culture c81-46A is slow growing and morphologically resembles melanocytes; c81-61 is an intermediately growing cell strain; c83-2C is a rapidly growing strain with the additional capacity of vertical growth in vitro; A375 is a commercially available cell line with well-described characteristics (ATTC).

To generate melanocyte cultures, two to five white people neonatal foreskins were placed in 0.25% trypsin at 4°C overnight. The tissues were scraped to recover the melanocytes, pooled and cultured in MCDB 153 (Sigma) medium containing cysteine (42 mg/l) and methionine (44.8 mg/l) with additions of 2% foetal calf serum, 0.3% bovine pituitary extract (Clonetics Corp., San Diego, CA, USA), 10 ng/ml

phorbol myristate-13-acetate, 2.0 mM calcium chloride, 5 μg/ml insulin and 0.1 mM 3-isobutyl-1-methyl-xanthine (Sigma). Each normal melanocyte pool contained the total yield of cells from two to five white people neonatal foreskins. The passage number used in these experiments was not greater than eight.

Both media contained penicillin(100 U/ml), streptomycin (0.1 mg/ml), bicarbonate (1.2 mg/ml) and phenol red indicator. No glutathione or mercaptoethanol was added to either media. In all experiments, cells were treated at 50–70% confluency and fresh media and drug added simultaneously for various times (24–72 h). Final density ranged from 70 to 90% confluency. No change was seen in media pH (7.6–7.8 was recorded).

Viability Assays

To control the oxygen concentration during viability studies, confluent cell cultures were incubated in a Billups-Rothenburg MIC-101 Hypoxia/Tissure Culture Incubator (Billups-Rothenburg Inc., Del Mar, CA, USA). A mixture of 95% oxygen and 5% carbon dioxide was pumped into the chamber and sealed in. The chamber was then placed in an incubator at 37°C. Every 24 h the chamber was opened to accommodate another cell culture for different time-points. Every time a new culture was added, more oxygen was re-pumped into the chamber. Control cultures were grown along side the chamber in the same incubator. Doubling the media volume had no effect on viability.

For the metal uptake studies, cell cultures were drugged with either $Cu(pdtc)_2$ or with $Zn(pdtc)_2$ at varying concentrations (0.1–1.0 μg/ml) for up to 72 h. The drugs were administered as DMSO solutions, and the maximum DMSO concentration was $<1\%$ (a non-toxic dose) in all experiments, and similar dose was applied to the control. Cultures were analysed for apoptosis by Annexin V binding according to the manufacturer's protocol (Pharmingen, San Diego, CA, USA). Briefly, cells were harvested, washed with 1X PBS and re-suspended in binding buffer. Of the cell solution, 100 μl was incubated with 5 μl of Annexin V (conjugated to FITC) and 15 μM of propidium iodide for 15 min at room temperature. Cells were analysed by flow cytometry using a Becton-Dickinson FACScan with Cell Quest software (Becton-Dickinson Biosciences, Mansfield, MA, USA). The proportion of apoptotic cells was estimated by the percentage of cells that stained positive for Annexin V while remaining impermeable to propidium iodide. Statistical calculations were made using Graphpad Prizm (Graphpad Software Inc., San Diego, CA, USA) to calculate the means of the data as well as the SEM values. The error bars shown are the SEM values of the means of the data.

RESULTS AND DISCUSSION

In a previous study from the Meyskens lab, melanoma cells in culture were shown to respond ineffectually against peroxide stress (8). Under glucose/glucose oxidase induced peroxide stress, melanocytes and several other cell types (keratinocytes, lung and oral carcinoma) were shown to effectively neutralize peroxide stress, generated by glucose oxidase (GOX), over a period of minutes. In contrast, several different strains of melanoma could not neutralize the stress, and a continued buildup of ROS was seen over time, as demonstrated by a ROS-sensitive chemiluminescent (CL) probe. Addition of catalase (CAT) decreased the CL signal, but addition of superoxide dismutase (SOD) paradoxically produced a several-fold increase in CL signal, implying that a high flux of superoxide was being generated. The differential response between melanocyte and melanoma suggested some essential change had occurred within the transformed cancer cells that rendered them incapable of neutralizing oxidative stress.

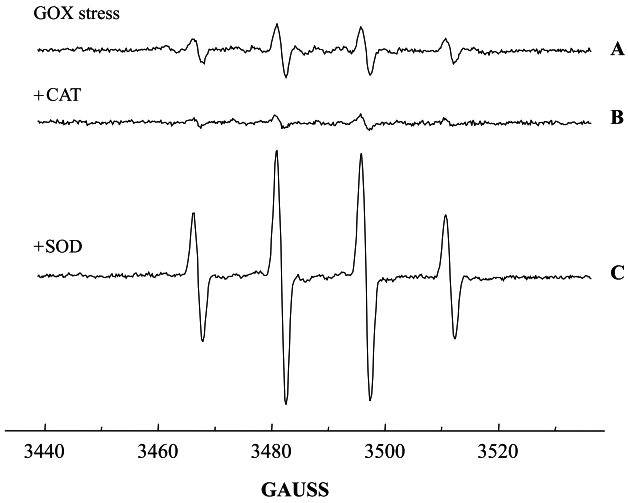

Fig. 1. EPR spectra of DMPO-OH concentrations obtained by trapping of ROS generated from melanoma cell line c81-46a: (**A**) after incubation with 20 U/ml GOX for 2 h prior to addition of DMPO to 32 mM; (**B**) addition of CAT (40 μg/ml) 15 min prior to DMPO; (**C**) addition of SOD (20 μg/ml) 15 min prior to DMPO.

To further test this unusual behaviour, the radical-trap DMPO was used to capture ROS generated by melanoma cells under similar GOX stress, results are shown in Fig. 1. 5,5′-dimethyl-pyrollidine N-oxide traps both the hydroxyl and superoxide radicals to form EPR active species, but the superoxide adduct is short-lived and decomposes to a more stable DMPO-OH adduct (quartet at $g = 2.0060$, $a(H) = a(N) = 1.49$ mT) over the timescale of this experiment (21). In one set of experiments, cultured melanoma c81-46a cells were incubated with glucose oxidase for 2 h before addition of DMPO. Spectra A denotes the ROS trapped after 15 min; for spectra B catalase (CAT) was added 15 min prior to DMPO; and in spectra C, superoxide dismutase (SOD) was added 15 min prior to DMPO. As in the CL experiments, CAT decreases the DMPO-OH signal, and a large increase was seen upon addition of SOD, implying a high flux of superoxide being generated by the peroxide stress.

ROS Generation by DHI-melanins

Preliminary studies suggested that metal ion binding increases melanin's ability to react with molecular oxygen to produce ROS by redox cycling. To test this, we presoaked electrodeposited poly DHI films anaerobically in Cu(II) and

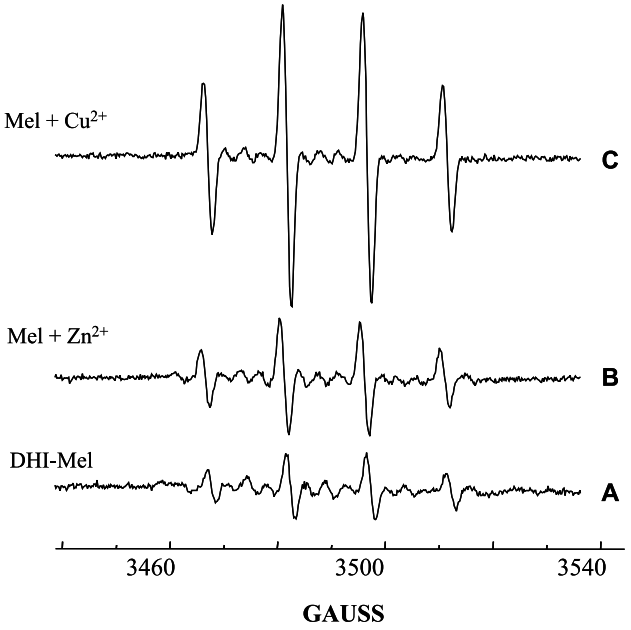

Fig. 2. Normalized DMPO-OH EPR signals from: (**A**) DHI-melanin films soaked in pH 7 buffer under air for 2 h prior to addition of DMPO to 32 mM; (**B**) same with DHI-melanin pretreated with Zn(II) salt; (**C**) same with DHI-melanin pretreated with Cu(II) salt, as described in the text.

Zn(II) salt solutions, then repeatedly washed and dried the films before exposing them to air-saturated solutions containing DMPO for 2 h before EPR measurement. The resulting spectra are from solutions after the withdrawal of the solid melanin films, therefore without interference from the characteristic melanin radical signal. The results, seen in Fig. 2, show that the DMPO-OH signal generated by poly DHI melanin is doubled by pretreatment with Zn(II), and enhanced six-fold by Cu(II) treatment.

We hypothesize that the CL and DMPO-OH signals generated by melanoma cells derive from exposed melanin and melanoid fragments within these cells. If so, we would expect a similar response to the addition of CAT and SOD. Fig. 3 gives the comparative results of CAT and SOD addition for a DMPO-OH generation using poly DHI films with and without metal-ion pretreatment. For these experiments, each signal is from an individual film soaked in DMPO solution for 2 h, with or without CAT or SOD, before measurement by EPR. To discount the influence of the electrode material on the reactivity of the deposited melanin, two materials were used: hydrophobic pyrolytic graphite (PG) and hydrophilic indium tin oxide (ITO). While the magnitude of the differences varies, the presence of CAT consistently diminishes the DMPO-OH signal, while that of SOD increases it; the one anomalous result was a very large increase in DMPO-OH signal seen for the Cu-treated film on ITO after addition of SOD. Overall, the similarity of the response in both chemical and cell culture studies suggests that both are of the same source, pro-oxidant melanin.

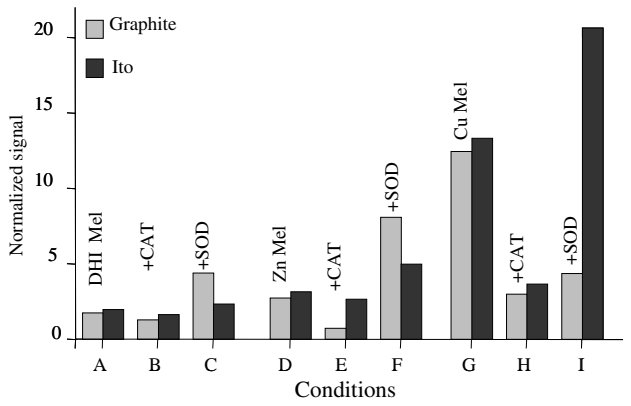

Fig. 3. Normalized intensities for DMPO-OH EPR signals generated from reaction of oxygen with melanin films on ITO and graphite, as described in text: (**A**) melanin; (**B**) melanin +catalase; (**C**) melanin +SOD; (**D**) Zn-treated melanin; (**E**) Zn-treated melanin + catalase; (**F**) Zn-treated melanin + SOD; (**G**) Cu- treated melanin; (**H**) Cu-treated melanin + catalase; (**I**) Cu-treated melanin + SOD.

Melanin's ability as a reductant complicates the DMPO trapping experiments, as melanin also reduces alkyl nitroxide radicals such as DMPO-OH (19). In anaerobic experiments, preformed DMPO-OH solutions exposed to poly DHI films lost over 10% of the EPR signal (data not shown). Smaller signal loss is predicted in aerobic experiments of Fig. 3, where O_2 should be in large excess to the DMPO-OH radical. There also remains some uncertainty over the types of ROS generated by the poly DHI films. The strong peroxide dependence suggests a direct formation of DMPO-OH by hydroxyl radicals generated from Fenton-like chemistry, but the unusual effect of SOD demonstrates a large flux of superoxide, which can rapidly disproportionate to peroxide (22).

Several recent reports have suggested that melanin initiates DNA breakage by hydroxyl radical generation (23, 24). To specifically assay hydroxy radicals, we exposed melanin films to supercoiled *pUC-18* plasmid DNA; when nicked or damaged by OH radicals, the integrity of the supercoiled DNA is lost, resulting in a distinctive shift in mobility during agar gel electrophoresis (25). The results of assays with and without Zn and Cu treatment are given in Fig. 4. On the timescale of the EPR experiments (2 h, data not shown), only the Cu-treated DHI-melanin showed significant DNA damage. On longer timescales (24 h), DHI-melanin itself is seen to cause observable breakage, which is enhanced by pretreatment with Zn(II), and greatly so by Cu(II). These results confirm the ability of poly DHI melanin to directly form cytotoxic hydroxyl radicals.

Melanoma Susceptibility to Oxygen

If exposed melanins within melanoma are as reactive as synthetic melanins, oxygen itself should be toxic to these cancer cells. It is known that high oxygen conditions are generally damaging to cells in culture (26), so to test this hypothesis a direct comparison was made of the viability of melanocyte and metastatic melanoma 81-46A cultures, grown in normal conditions and under 95% O_2 5% CO_2 over a 3-d period. Figure 5 shows combined data from multiple sets of time-course studies. In agreement with our supposition, melanoma are much more susceptible to oxygen

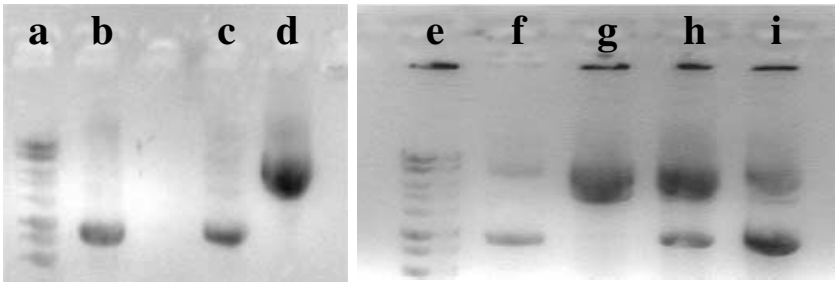

Fig. 4. Gel separations of plasmid DNA showing hydroxy radical damage; left gel: (**a**) ladder; (**b**) untreated plasmid DNA; (**c**) DNA exposed to PG electrode; (**d**) DNA exposed to Cu-treated melanin film; right gel: (**e**) ladder; (**f**) untreated DNA; (**g**) DNA exposed to Cu-treated melanin; (**h**) Zn-treated melanin; (**i**) DHI-melanin alone.

than melanocytes; one-tailed *t*-tests of the means of the data show that the viability and cell death are significantly different under high and low oxygen conditions at 48 h ($P < 0.0002$). Similar results were obtained for melanoma cell lines c83-2C, c81-61 and A375 (data not shown). In all cases the decrease in viability begins after the first 24 h; over the 3-d time course there is some increase in apoptosis, but cell death is mainly manifest as necrosis. Because of the increased oxidative conditions, apoptotic cells that die early in the incubation period may develop necrotic elements, as was seen in a study of UV-induced cell death in the human squamous cell carcinoma (27).

Melanoma Susceptibility to Metal Uptake Drugs

Melanoma cells have a demonstrated sensitivity to certain metals, perhaps attributable to a pro-oxidant response of cytosolic melanin fragments upon metal-uptake. Therefore, melanomas may be susceptible to drugs which increase metal-uptake. Dithiocarbamates (DTCs) are chelators derived from CS_2, which form strong lipophilic complexes with metal ions that passively diffuse through cell membranes. Slater has shown that DTCs act as pro-oxidants by increasing Cu uptake into cells (28, 29). A number of other studies have demonstrated that the cytotoxicity of pyrrolidine dithiocarbamate (pdtc) depends on the availability of metal ions such as Cu(II) and Zn(II) in the media (30, 31).

We find that the pdtc complexes of Cu(II) and Zn(II) are toxic to melanoma at low dosages over a 3-d period, Figs 6 and 7. The Zn(II) adduct has more limited toxicity under normal conditions, viability drops by *c*. 20% at 0.1 μg⁄ml ($P < 0.0087$ compared with control), and halves again under high O_2 conditions ($P < 0.0002$ compared with control). The Cu(II) adduct is more active, melanoma viability drops by a third at 0.1 μg⁄ml drugging under air ($P < 0.043$ compared with control); under high oxygen conditions viability drops by 75% ($P < 0.0002$ compared with control).

Contrary to our expectations, the combination of metal-uptake drug with high oxygen seems additive rather than synergistic. The toxicity of $Zn(pdtc)_2$ manifests mainly as necrosis, as does that of high oxygen stress. In contrast, $Cu(pdtc)_2$ induces large increases in apoptosis, which may

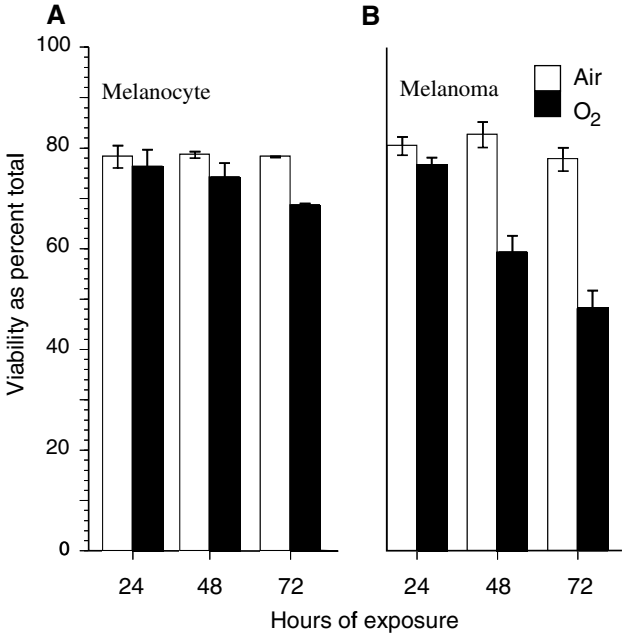

Fig. 5. Cell viability over time under air or 95% O_2⁄5% CO_2 controlled atmosphere: (**A**) melanocytes, average of three sets of experiments; (**B**) melanoma cell line c81-46a, average of seven sets.

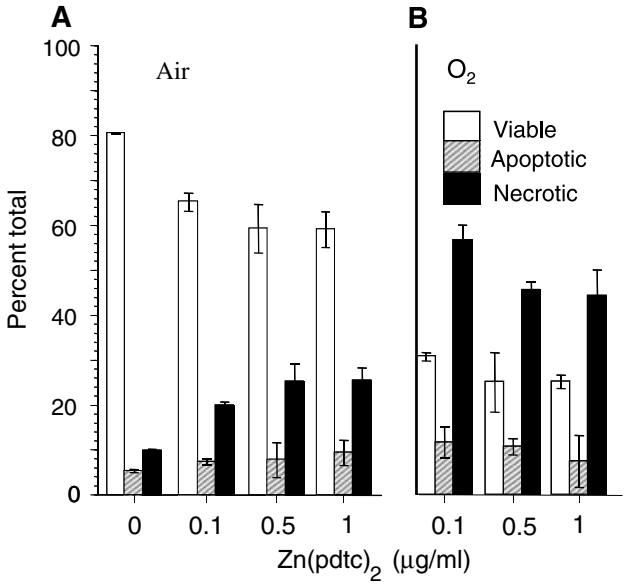

Fig. 6. Melanoma cell line c81-46a viability at 72 h after treatment with varying concentrations of $Zn(pdtc)_2$ (**A**) under air; (**B**) 95% O_2⁄5% CO_2. Average of two sets; the viability for high oxygen exposure in the absence of $Zn(pdtc)_2$ are well within the range for the data presented in Fig. 5.

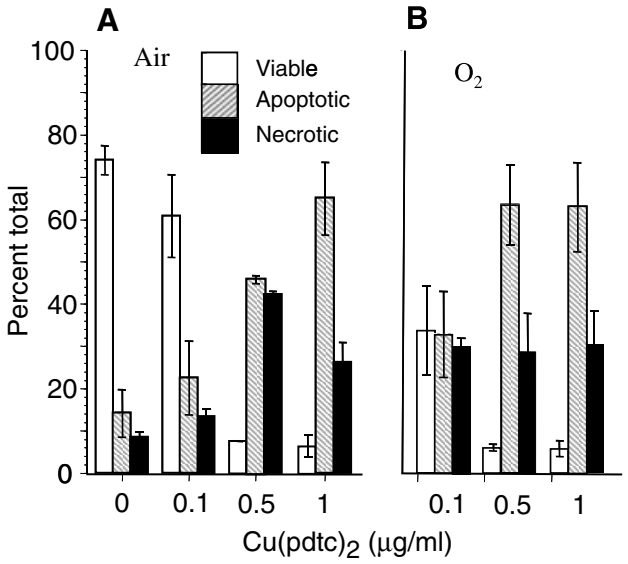

Fig. 7. Melanoma cell line c81-46a viability at 72 h after treatment with varying concentrations of $Cu(pdtc)_2$ (**A**) under air; (**B**) 95% O_2/5% CO_2. Average of four sets; the viability for high oxygen exposure in the absence of $Cu(pdtc)_2$ are well within the range for the data presented in Fig. 5.

indicate a different mechanism of action. In this regard, the activity of $Cu(pdtc)_2$ resembles that of disulfiram, the disulphide form of diethyl dithiocarbamate, whose toxicity correlates to changes in cellular glutathione redox status (32).

As the more active compound, the toxicity of $Cu(pdtc)_2$ was tested against normal melanocytes; the results are given in Fig. 8. Here the effect of the drug is much diminished, with a maximum loss of *c*. 30% under both high and low O_2 conditions. Interestingly, melanocyte cultures grown with the lower 0.1 μg/ml dosage showed a significant increase in viability ($P < 0.0337$ compared with control). Similar complex responses to dithiocarbamates have been seen for

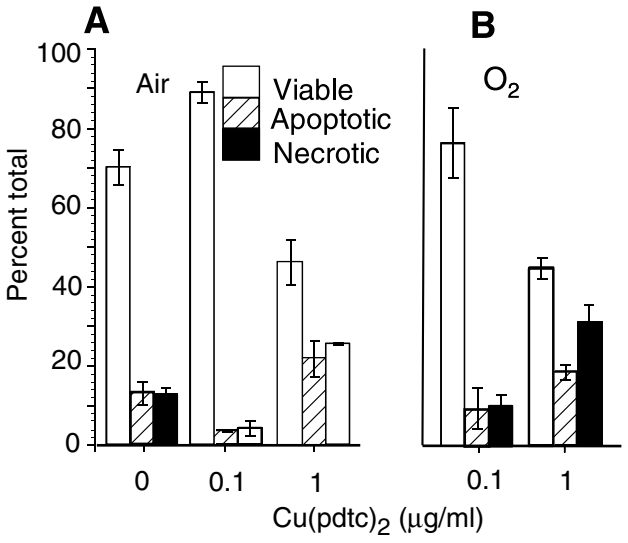

Fig. 8. Melanocyte viability at 72 h after drugging for 72 h with $Cu(pdtc)_2$: (**A**) in air; (**B**) in 95% O_2/5% CO_2. Average of two sets.

other cell lines, perhaps because of their antioxidant abilities (29). As the lower dosage is highly toxic to melanoma under high O_2, a combination treatment may be envisioned that would selectively target melanomas.

CONCLUSION

This work is the first step towards the possibility of using melanin redox-cycling to selectively target melanoma. We have previously postulated that the sensitivity of melanoma cell cultures to oxidative stress derives from melanin-generated superoxide (8); the present results suggest that hydroxy radicals may also play a role. Using synthetic melanin, we demonstrated that its reaction with oxygen produces hydroxy radicals, and that this reactivity is increased by the binding of Zn and Cu. In agreement with our hypothesis that such melanin-based reactivity should be more pronounced in melanoma than melanocytes, we have demonstrated that melanoma have an enhanced susceptibility to oxygen. We have also shown antimelanoma activity for Zn and Cu dithiocarbamate complexes, which are known to transport the metal ions into cells. Ultimately, we hope to exploit the unique chemistry of the melanin pigment as a means of targeting melanoma in vivo.

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REFERENCES

1. Helmbach H, Rossmann E, Kern MA, Schadendorf D. Drug resistance in human melanoma. Int J Cancer 2001;93:617–622
2. Jimbow K, Iwashina T, Alena F, Yamada K, Pankovich J, Umemura T. Exploitation of pigment biosynthesis pathway as a selective chemotherapeutic approach for malignant melanoma. J Invest Dermatol 1993;100:231S–S238
3. Riley PA, Cooksey CJ, Johnson CI, Land EJ, Latter AM, Ramsden CA. Melanogenisis-targeted anti-melanoma pro-drug development: effect of side-chain variations on the cytotoxicity of tyrosinase-generated ortho-quinones in a model screening system. Eur J Can 1997;33:135–143
4. Borovansky J, Mirejovsky P, Riley PA. Possible relationship between abnormal melanosome structure and cytotoxic phenomena in malignant melanoma. Neoplasma 1991;38:4–10
5. Borovansky J. Properties of melanosomes and their exploitation in the diagnosis and treatment of melanoma. Mel Res 1993;3:181–186
6. Meyskens FL, Buckmeier JA, McNulty SE, Tohidian NB. Activation of nuclear factor-kappa B in human metastatic melanoma cells and the effect of oxidative stress. Clin Canc Res 1999;5:1197–1202
7. Meyskens FL, Farmer PJ, Fruehauf J. Redox regulation in human melanocytes and melanoma. Pigment Cell Res 2001;14:148–154
8. Meyskens FL, Van Chau H, Tohidian N, Buckmeier JA. Luminol-enhanced chemiluminescence response of human melanocytes and melanoma cells to hydrogen peroxide stress. Pigment Cell Res 1997;10:184–189
9. Meyskens FL, McNulty SE, Buckmeier JA, Tohidian NB, Spillane TJ, Kahlon RS, Gonzalez RI. Aberrant redox regulation in human metastatic melanoma cells compared to normal melanocytes. Free Rad Biol Med 2001;31:799–808
10. Bedrick AE, Ramaswamy G, Tchertkoff V. Histochemical determination of copper, zinc, and iron in some benign and malignant tissues. Am J Clin Pathol 1986;86:637–640
11. Borovansky J, Mirejovsky P, Riley PA. The effect of divalent cations on Cloudman melanoma cells. Eur J Cancer Clin Oncol 1983;19: 91–99

12. Borovansky J, Blasko M, Siracky J, Schothorst AA, Smit NPM, Pavel S. Cytotoxic interactions of Zn^{2+} in vitro: melanoma cells are more susceptible than melanocytes. Mel Res 1997;7:449–453
13. Sarzanini C, Mentasti E, Abollino O, Fasano M. Metal ion content in *Sepia officinalis* melanin. Mar Chem 1992;39:243–250
14. Gidanian S, Farmer PJ. Redox behavior of melanins: direct electrochemistry of DHI-melanin and its Cu and Zn adducts. J Inorg Biochem 2002;89:54–60
15. Szpoganicz B, Gidanian S, Kong P, Farmer PJ. Metal binding by melanins: studies of colloidal DHI-melanin, and its complexation by Cu (II), and Zn (II) ions. J Inorg Biochem 2002;89:45–53
16. Korytowski W, Sarna T. Bleaching of melanin pigments: role of copper ions and hydrogen peroxide in autooxidation and photooxidation of synthetic dopa-melanin. J Biol Chem 1990;285:12 410–12 416
17. Sarna T, Duleba A, Korytowski W, Swartz H. Interaction of melanin with oxygen. Arch Biochem Biophys 1980;200:140–148
18. Sarna T, Swarz HM. Interactions of melanin with oxygen (and related species). In: Scott G. Atmospheric Oxidation and Antioxidants, Vol. III. Amsterdam: Elsevier Science Publishers; 1993. pp. 129–165
19. Sarna T, Korytowski W, Sealy RC. Nitroxides as redox probes of melanins: dark-induced and photoinduced changes in redox equilibria. Arch Biochem Biophys 1985;239:226–233
20. Chung CT, Miller RH. A rapid and convenient method for the preparation and storage of competent bacteria cells. Nucl Acids Res 1988;16:3580
21. Finkelstein E, Rosen GM, Rauckman EJ, Paxton J. Spin trapping of superoxide. Mol Pharm 1979;16:676–685
22. Grisham MB. Role of reactive oxygen metabolites in pathophysiology. In: Grisham MB. Reactive Metabolites of Oxygen and Nitrogen in Biology and Medicine. Austin: R.G. Landes Company; 1992. Chap. 7.
23. Husain S, Hadi SM. Strand scission in DNA induced by L-DOPA in the presence of Cu (II). FEBS Lett 1995;364:75–78
24. Husain S, Hadi SM. DNA Breakage by L-DOPA and Cu (II): breakage by melanin and bacteriophage inactivation. Mut Res Fund Mol Mech 1998;397:161–168
25. Hermes-Lima M, Nagy E, Ponka P, Schulman HM. The iron chelator pyridoxal isonicotinyl hydrazone (PIH) protects plasmid pUC-18 DNA against OH-mediated strand breaks. Free Rad Biol Med 1998;25:875–880
26. Spitz DR, Elwell JH, Sun Y, Oberley LW, Oberley TD, Sullivan SJ, Roberts RJ. Oxygen toxicity in control and hydrogen peroxide-resistant Chinese hamster fibroblast cell lines. Arch Biochem Biophys 1990;279:249–260
27. Schindl A, Klosner G, Honigsmann H, Jori G, Calzavara-Pinton PC, Trautinger F. Flow cytometric quantification of UV-induced cell death in a human squamous cell carcinoma-derived cell line: dose and kinetic studies. J Photochem Photobiol B 1998;44:97–106
28. Nobel CSI, Kimland M, Lind B, Orrenius S, Slater AFG. Dithiocarbamates induce apoptosis in thymocytes by raising the intracellular level of redox-active copper. J Biol Chem 1995;270:26 202–26 208
29. Orrenius S, Nobel CSI, Bandendobbelsteen DJ, Burkitt MJ, Slater AFG. Dithiocarbamates and the redox regulation of cell death. Biochem Soc Trans 1996;24:1032–1038
30. Chung KC, Park JH, Kim CH, Lee HW, Sato N, Uchiyama Y, Ahn YS. Novel biphasic effect of pyrrolidine dithiocarbamate on neuronal cell viability is mediated by the differential regulation of intracellular zinc and copper ion levels, NF-kappa B, and MAP kinases. J Neurosci Res 2000;59:117–125
31. Erl W, Weber C, Hansson GK. Pyrrolidine dithiocarbamate-induced apoptosis depends on cell type, density, and the presence of Cu^{2+} and Zn^{2+}. Am J Phys Cell Phys 2000;278:C1116–C1125
32. Cen D, Gonzalez RI, Buckmeier JA, Kahlon RS, Tohidian NB, Meyskens FL. Disulfiram induces apoptosis in human melanoma cells – a redox related process. Mol Canc Ther 2002;1:197–204