Published in final edited form as:

Arch Biochem Biophys. 2014 December 1; 563: 71–78. doi:10.1016/j.abb.2014.07.033.

TARGETING MELANOCYTE AND MELANOMA STEM CELLS BY **8**-HYDROXY-**2**-DIPROPYLAMINOTETRALIN

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Abstract

Monobenzyl ether of hydroquinone (MBEH) is cytotoxic towards melanocytes. Its treatment efficacy is limited by an inability to eradicate stem cells. By contrast, 8-hydroxy-N,N-dipropyl-2-aminotetralin (8-DPAT) affects melanocyte stem cell survival. MBEH and 8-DPAT were added to melanocytes and melanoma cells to compare cytotoxicity. Stem cell content among viable cells was determined by fluorocytometry using markers CD34, Pax3, and CD271. Immunostaining was used to identify stem cells in skin explants treated with MBEH or 8-DPAT *ex vivo*. Mice were exposed to MBEH or 8-DPAT and scanned for depigmentation before harvesting skin. MBEH exposure prompted a relative increase in stem cells among cultured melanocytes and melanoma cells, as treatment preferentially eliminated differentiated cells and spared the stem cells. Viability of this remaining, enriched stem cell population was however rapidly reduced by exposure to 8-DPAT within melanocyte and melanoma cell cultures. In human skin explants, the abundance of melanocyte stem cells was also visibly reduced after 8-DPAT treatment, in contrast to tissue exposed to MBEH. Meanwhile, significant depigmentation of the mouse pelage and loss of differentiated melanocytes was observed *in vivo* in response to topical application of MBEH, but not 8-DPAT. Prolonged application of the latter agent instead appeared to effectively reduce the abundance of melanocyte stem cells in the dermis. This furthers the idea that MBEH and 8-DPAT target complementary cell populations. Results indicate that combination treatment may demonstrate superior therapeutic activity by eliminating both differentiated and tumor initiating populations.

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Keywords

MBEH; 8-DPAT; melanocyte; melanoma; stem cells; viability; pigmentation

INTRODUCTION

The incidence of melanoma has gradually increased since the 1980s at a rate of approximately 2.8% annually (1). There were more than 75,000 new cases in the United States in 2013, accounting for almost 5% of all new cancers. It is estimated that more than 920,000 people are living with melanoma in the U.S, where the incidence is about 21 per 100,000 per year (2). The incidence of melanoma in the United Kingdom and Australia is 12.6 and as high as 46.7 per 100,000, respectively (1). Approximately 20% of those diagnosed with cutaneous malignant melanoma will die secondary to metastatic disease (3).

Conventional chemotherapy is largely ineffective for the treatment of metastatic melanoma. There has been increased focus on the use of immunotherapy agents which demonstrate much greater success in disease regression. High-dose Interleukin-2 therapy was approved by the FDA in 1998 for metastatic disease, but response rates were less than 20% (4). However, approximately 8% of treated patients do achieve complete disease remission. Cytotoxic T-lymphocyte antigen 4 (CTLA-4) has been shown suppress T-cell activation. Antibodies to CTLA-4 potentiate the immune response against tumor cells. A monoclonal antibody against CTLA-4 has demonstrated improved survival in patients with stage 3 and 4 melanoma (5), and in 2011 became the first new FDA-approved treatment for advanced melanoma in more than 10 years. The approval of BRAF inhibitors for melanoma treatment is revealing some spectacular remissions in late stage melanoma patients, however, these tumors do recur (6). Clearly, it is important to understand the process of drug resistance that evolves within tumors under these circumstances.

It was previously believed that melanoma arose from mature melanocytes, but growing evidence suggests that a subpopulation of melanoma stem cells is mostly responsible for disease initiation and proliferation (7). Melanoma stem cells demonstrate self-renewal ability and give rise to the full heterogeneous composition of the tumor. Only a unique subset of tumor cells possesses these qualities, though the proportion of stem cells reportedly found within individual tumors is inconsistent, ranging from 0.1% to 53% in several studies (8, 9). Nevertheless, there is much interest in identifying markers of melanoma stem cells, as the ability to specifically target these cells may lead to development of new therapies. CD271 represents the low-affinity receptor for neural growth factor and is a neural crest stem cell marker found on human multipotent melanoma cells. These stem cells have been shown to establish the full heterogeneity of parental melanoma when xenotransplanted to nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (9). CD34 has likewise been identified as a marker of melanoma progenitor cells in mouse models (10). Melanocyte stem cells provide pigmented cells during tissue homeostasis. Melanoma is thought to arise from transformed mature melanocytes, melanoma-specific stem cells, or both (7). Melanocyte stem cells are found in hair follicles primarily. Similar to melanoma stem cells, they are influenced by other cell types and are

affected by various factors that promote or restrict multipotency and self-renewal (11). Melanocyte stem cells can be found in hair follicles, but also derive from neural crest cells in the dermis (12).

Neural crest cells, after migrating from the neural plate, can differentiate into a wide array of cell types, including bone, cartilage, and melanocytes. Melanoblasts derive from glial-melanoblast progenitors, whose fate is in part determined by the Wnt signaling pathway (13). Many important genes have been identified as crucial in the development of melanocytes. Pax3, a gene in the paired box family of transcription factors, also plays a role in melanoblast development, possibly by balancing differentiation to melanocytes, versus maintaining an undifferentiated state (13). Mutations in Pax3 are responsible for the Waardenburg syndrome phenotype, a rare auditory-pigmentary syndrome. c-Kit is a tyrosine kinase receptor which, when ligated, activates signaling implicated in melanocyte differentiation, migration, and differentiation. Multiple important signaling pathways work downstream from the Kit receptor, including PI3K and RAF/MEK/ERK pathways (14). Though no single marker is currently universally accepted as an identifier of all melanocyte or melanoma stem cells, their typical location and a combination of CD271, Pax3 and CD34 expression may be considered suitable to identify these populations under different conditions.

Mutations in the oncogene BRAF, which is dependent on RAF/MEK/ERK, are common in melanoma, and drugs targeting this pathway have recently been FDA approved for metastatic disease (6). Studies of the zebrafish model have led to a deeper understanding of melanocyte biology and the complexity of these pathways. Melanocytes are visible both in the embryo and in the mature zebrafish. This transparent quality, in addition to their small size and ability to produce numerous embryos, makes zebrafish an ideal model for large-scale screening studies of small molecules. In this regard, 8-hydroxy-2-dipropylaminotetralin (8-DPAT) has been shown to modify kit-dependent melanocyte migration and survival in drug-screening studies on zebrafish, resulting in a markedly reduced number of migrated melanocytes versus when compared to the untreated zebrafish population (14). Interestingly, in response to 8-DPAT the migrated melanocytes are smaller and appear to fragment similar to that seen in zebrafish treated with (2-morpholinobutyl) -4-thiophenol (MoTP), a melanocytotoxic drug dependent on tyrosinase activity (14,15).

Radiolabeled 8-DPAT is commonly used to delineate the expression and distribution of 5-hydroxytryptophan (serotonin) 1A receptors in human brain. The drug is also used as a 5HT1a receptor agonist to study effects on serotonin reuptake inhibition and prevention of depression (16, 17). Taken together, the role of 8-DPAT is best established in relation to neuronal function. These studies suggest that responses to 8-DPAT observed in zebrafish may well translate to mammals including mice and men.

Targeting of adult melanocytes is readily accomplished by monobenzyl ether of hydroquinone (MBEH), an FDA-approved melanocytotoxic drug for depigmentation therapy in vitiligo. Our previous works have explored how MBEH mediates depigmentation, demonstrating that this compounds induces melanocyte necrosis while another depigmenting compound, 4-TBP (4-tertiary butyl phenol), induces cell death by apoptosis (15). We have

subsequently shown that MBEH treatment leads to a CD8 T-cell response which targets sites distant from the site of topical application, making it a potentially useful agent for melanoma prophylaxis (18). As MBEH-induced depigmentation likely requires tyrosinase which is not expressed by undifferentiated stem cells, we hypothesized that treatment will leave a population of stem cells that is resistant to treatment, and agents targeting the stem cell population are thus predicted to enhance the efficacy of MBEH treatment in either disease.

Throughout our studies, we have engaged antibodies to CD34, CD271 and Pax3 to identify the stem cell population. We studied the potential of 5HT-1A agonist 8-DPAT to affect melanocyte and melanoma stem cell viability *in vitro* by MTT assays using a range of 8-DPAT concentrations, evaluated morphologic changes induced by 8-DPAT exposure, assessed topical application of 8-DPAT to mouse and human skin *in vivo* and in organotypic explant cultures, respectively, and evaluated its effects on the abundance of melanocyte and melanoma by scanning and immunohistochemistry. Taken together, these experiments outline the opportunities for stem cell targeting presented by 8-DPAT exposure and its potential synergism with MBEH for the treatment of pigmentation disorders.

MATERIALS AND METHODS

Mouse Model

C57BL/6J mice from The Jackson Labs (Bar Harbor, Maine, USA) were used for all of the depigmentation and organotypic culture experiments. In the topical treatment study 5 mice per group were used. All experiments were approved by Loyola University Medical Center's Institutional Animal Care and Use Committee.

Preparation of Bleaching Agents and Treatment

Both 8-DPAT (R-(+)-8-hydroxy-DPAT) (Sigma-Aldrich, St. Louis, Missouri, USA) and MBEH (Sigma) were prepared as stock solutions of 250mM in 1:5 mixture of DMSO and 70% EtOH respectively. This 1:5 DMSO-EtOH mixture was also used as the vehicle control. These stocks were diluted to a final concentration of 50mM to 250mM to treat organotypic cultures and further diluted to 50μM to 500μM to treat cell cultures. Additional stocks of 900mM 8-DPAT (8-hydroxy-DPAT hydrobromide) (Tocris, Bristol, United Kingdom) and 450mM MBEH (Sigma) were prepared for topical treatment of mice. After these stocks were diluted 1:1 with Eucerin lotion (Beierdorf, Wilton, Connecticut, USA), resulting in 450mM and 225 mM concentrations, they were topically applied in 100μl volumes to the hair free abdominal skin of C57BL/6 mice. Mice were placed under anesthesia during the hair removal process, in which Nair (Church and Dwight Co., Princeton, New Jersey, USA) was used, as well as for each topical application. Twenty-four hrs after hair removal the Eucerin mixtures were added and massaged into the skin. Animals were treated every other day for three weeks and Naired as needed. Three weeks post treatment, after the last scan the mice were humanely euthanized. Treated ventral and untreated dorsal skin from each mouse was harvested, snap frozen, and stored for immunostaining.

Measuring Depigmentation

To evaluate the amount of depigmentation in response to the topical treatments with vehicle, 8-DPAT, or MBEH a flatbed scanner was used to take images and Adobe Photoshop CS6 13.0 (Adobe Systems Inc., San Jose, California, USA) was used to analyze those images. Prior to the initial Nair treatment, mice were placed under anesthesia and ventrally scanned. The mice were scanned again 3 weeks post treatment for assessment of depigmentation. Equal amounts of pixels from each mouse were selected, converted to grayscale, and color inverted. The pixels were then measured for average luminosity and compared to that of the untreated mice in an Excel (Microsoft, Seattle, Washington, USA) spreadsheet.

Organotypic culture of treated human skin

Human explants were acquired from otherwise discarded neonatal foreskin samples. Biopsies of approximately 6 mm diameter were then placed, dermis side down, onto a 0.4µm pore size membrane insert of a 12-well sterile culture dish (Corning Incorporated, Teterboro, New Jersey, USA). Into the bottom well, 400µl of media was added (Dulbecco's Modified Eagle's Medium, 10% heat-inactivated serum, and Antibiotics- Antimycotics mixture) and the tissue was incubated at 37°C, 5% CO₂ overnight. The next day, 10µl of MBEH, 8-DPAT, or vehicle at concentrations up to 250mM were pipetted onto the epidermis, and tissues were incubated for 48 hours. Tissues from 3 donors were then harvested and snap frozen in optimal cutting temperature compound (OCT) (Sakura Finetek USA, Torrance, California, USA) and stored at -80°C for later use.

Immune Single and Double Staining

OCT embedded human and mouse skin samples were used for sectioning and staining. Eight µm cryostat sections were cut and air dried for 1hr, then fixed in cold acetone for 10min. Fixed slides were stained immediately or stored at -20°C for later use. Mouse skin sections were indirectly immunostained using primary antibodies to tyrosinase-related protein-1 (Trp-1; clone Ta99, mouse α-human/mouse IgG2a, Covance, Princeton, NJ) or Pax3 (rabbit anti-human/mouse/rat polyclonal IgG, Bioss, Woburn, MA). After washing, slides were incubated with horseradish peroxidase-labeled goat anti-mouse IgG2a (Southern Biotechnologies, Birmingham, AL) or with alkaline phosphatase-labeled goat anti-rabbit IgG, respectively. Slides were then coverslipped using gelvatol mounting medium (Dakopatts, Glostrup, Denmark) and imaged.

Human tissues were stained using primary antibodies to CD271 (C40-1457, mouse α-human, IgG1, BD Biosciences, San Jose, CA) and to Pax3 (rabbit α-mouse/human/rat polyclonal IgG, Bioss) followed by phycoerythrin-labeled secondary goat α-mouse IgG1 (Southern Biotechnologies) and Dylight-labeled donkey α-rabbit IgG (Biolegend, San Diego, CA) respectively. These were then cover slipped using 4',6-diamidino-2-phenylindole-containing mounting medium (Invitrogen, Grand Island, New York), imaged on an Olympus BX41 fluorescence microscope (Center Valley, PA), colored and merged using Adobe Photoshop CS6 13.0 (San Jose, CA).

Cytotoxicity assay

Cytotoxicity of the bleaching phenols towards cells in culture was determined through a commercially purchased MTT assay (Bioassay system, Hayward, California, USA). Per the manufacturers' instructions, 20,000 cells were plated per well in triplicate into 96-well flat bottom plates. Culture plates were incubated at 37°C for 24hrs to allow cell adherence. The cells were then treated with 250µM or 500µM MBEH or 8-DPAT, vehicle alone, or perm buffer to represent total lysis. Plates were incubated at 37°C and 5% CO₂ for 24 hrs., before adding 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) and incubating for another 4hrs. Live cells are able to convert tetrazole into the purple dye formazan, which is dissolved in solubilization buffer prior to quantification. The absorption read by a plate reader (BMG Labtech, Inc., Durham, North Carolina, USA) at 570nm is directly proportional to the number of living cells. Raw data from the plate reader were analyzed and compared to control samples to assess cell viability.

Stem Cell Identification

In order to test the effect of bleaching phenols on both differentiated and undifferentiated melanocyte or melanoma cell populations, a set of markers was selected for stem cell identification. CD34 is a widely accepted human stem cell marker and antibodies to CD34 (clone 581, mouse α-human, IgG1, BD Biosciences, San Jose, CA) were used as a marker for undifferentiated melanoma cells and melanocytes, combined with antibodies to CD271 (NGFRp-75) (clone C40-1457, mouse α-human, IgG1, BD Biosciences). In order to ensure that the CD34⁺, CD271⁺ cells were of the melanocyte lineage, antibodies to Pax3 (rabbit anti-human/mouse/rat IgG, Bioss, Woburn, MA) were included for immunostaining and fluorocytometric analysis.

RESULTS

MBEH but not 8-DPAT induces physiologic changes in primary melanocytes

Gross morphologic changes in response to bleaching agents MBEH and 8-DPAT were followed by adding the agents to the cell culture media for 24 hrs. In Fig. 1 it can be observed that even at the lower, 250 µM concentration of MBEH a significant number of cells have retracted dendrites, have detached from the culture flask and have undergone necrosis among treated melanocytes, whereas 624.38 melanoma cells remain relatively unaffected by the treatment. This is in concordance with our results published previously (15). By contrast, at the higher 500 µM concentration of 8-DPAT melanocytes remain relatively unaffected. In fact, both melanocytes Mf12386 in first passage and 624.38 melanoma cells retained a similar morphology in presence of 8-DPAT, although the number of cells appeared to be slightly reduced in response to 8-DPAT from primary melanocytes only. In conclusion, 250 µM MBEH is visibly cytotoxic towards melanocytes but not melanoma cells in 24 hrs but after that same treatment period, cells of either source remain viable in presence 500 µM 8-DPAT. Images are all represented at the same magnification for comparison.

MBEH and 8-DPAT reduce viability in melanocyte and melanoma cell cultures

Melanocytes from multiple donors were included at low passage numbers (max. 5) in a comparison of viability to PIG1 immortalized melanocytes at passage 51, 3 melanoma cell lines of mouse (B16) or human origin, and unrelated human T cell lymphoma line Jurkat. As observed in Fig. 2a, primary and immortalized melanocyte viability was markedly reduced following treatment with MBEH, remaining at approximately 50% in response to 250 μ M MBEH for 24 hrs and further reduced by >80% in response to 500 μ M concentrations for 24 hrs when compared to treatment with vehicle alone. Some decrease in viability was also observed among treated melanoma cells, amounting to approximately 50% reduced viability in response to 500 μ M MBEH treatment. Unrelated Jurkat cells were least sensitive to MBEH treatment. In Fig. 2b it can be observed that contrary to MBEH treatment, exposure to 8-DPAT induced only approximately 20% cytotoxicity in response to 500 μ M exposure among melanocytes and approximately 10% cytotoxicity towards melanoma cells at the same concentration added for 24 hrs. Taken together, these results indicated that viability was most affected by MBEH treatment of melanocytes but that both primary and malignant melanocytes were sensitive to high concentration of MBEH, and either cell type was moderately sensitive to 8-DPAT as well, with unrelated Jurkat cells being least sensitive. We have previously similarly shown that treatment with high concentrations of bleaching phenols is toxic to a broad range of cells types and cannot be considered selective for melanocytes (18).

8-DPAT affects stem cell viability among melanocytes and melanoma cells

In zebrafish, 8-DPAT was found to affect the viability, migration and differentiation of melanocyte stem cells (14). Here we examined whether 8-DPAT can similarly eradicate stem cells among human melanocytes and melanoma cell cultures. For this work, we define stem cells as those that express each of Pax3, CD34, and CD271, which have been individually identified as markers of melanoma and melanocyte stem cells (10,19-23). To determine the effect of 8-DPAT on stem cell viability among monocultures *in vitro*, both cell types were treated with or without 500 μ M 8-DPAT for 24 hrs. The surviving stem cell population was determined by fluorocytometric analysis, quantifying the Pax3⁺ CD34⁺ CD271⁺ populations among viable cells as shown in Fig. 3a and b. Compared to untreated samples where approximately 2% stem cells were detected among viable cells for both melanocytes and melanoma cells, the stem cell content was reduced by 30.5% (SD=9.99, N=3, p=0.003) and 35.6% (SD=13.9, N=4, p=0.004) after 24 hrs for melanocytes and melanoma cells in culture, respectively. Moreover, Fig. 3c shows that treatment with 250 μ M MBEH alone will markedly enrich the stem cell population to >17% among remaining, viable cells whereas treatment with a combination of MBEH and 500 μ M 8-DPAT illustrates the potent anti-stem cell effect of 8-DPAT, reducing the percent stem cells from 17.3% in presence of MBEH alone to 5.6% among remaining viable cells undergoing the combination treatment. These data are congruent with a cytotoxic effect of 8-DPAT on stem cells *in vitro*, and support the concept that stem cell targeting by 8-DPAT in a zebrafish model translates to a similar effect on mammalian melanocyte stem cells as well as on stem cells that can give rise to malignant melanoma tumors. The 24 hr time period of treatment is chosen to

retain a population of viable cells among all cultures at concentrations of interest, yet it may be extrapolated that the majority of stem cells can be eliminated upon extended treatment.

Topical application of 8-DPAT but not MBEH reduced melanocyte stem cell abundance in human skin explants

Given the remarkable effects of 8-DPAT on melanocyte and melanoma stem cells in culture, topical application to human skin was next examined to understand whether the agent can penetrate the epidermis and selectively eliminate melanocyte stem cells among other cell types present in normal human skin. Organotypic culture of human skin tissue was initiated and 10 μ l of up to 250mM MBEH or DPAT solutions were pipetted topically 48 hrs before snap-freezing the treated and untreated tissue samples. Cryosections prefixed in acetone were immunofluorescently stained for expression of Pax3 and CD271. Sections were counterstained with DAPI. Observing between 50-100 PAX3+/CD271+ stem cells per mm² in untreated neonatal skin from 3 donors, after 48 hours, tissue treated with 8-DPAT contained approximately 40% fewer double positive cells per mm², whereas MBEH-treated skin revealed no significant change in abundance of double positive melanocyte stem cells compared to vehicle treated skin. An example staining is shown in Fig. 4, representing 48 hr treatment with a 250 mM concentration of 8-DPAT. After 48 hours, tissue treated with 250-500 mM 8-DPAT contained significantly fewer PAX3+/CD271+ stem cells per mm² versus vehicle treated skin, decreasing this double-positive stem cell population by 39% on average (p=0.036). MBEH-treated skin revealed no significant change in abundance of double positive melanocyte stem cells (mean=87.8, SD=10.5, N=3) compared to vehicle (mean=78.3, SD=11.7, N=3). Because MBEH is known to target mature melanocytes whereas Pax3 serves as a survival marker, this result was to be expected (17). These data lead to the expectation that MBEH and 8-DPAT will target different subpopulations *in vivo*. Whereas MBEH preferentially targets differentiated melanocytes, stem cells are clearly more effectively targeted by 8-DPAT in human skin explants. Furthermore, these results highlight the ability of 8-DPAT to penetrate the skin and target melanocyte stem cells in human tissue. This suggests that this agent can be effective upon topical application, adding to the therapeutic value of this otherwise expensive drug for the treatment of melanoma, and it supports its use for the permanent elimination of melanocytes from skin.

Depigmentation of mouse pelage is seen with MBEH but not 8-DPAT treatment

MBEH is an established depigmentation agent used by patients with extensive vitiligo to depigment any remaining areas and obtain an even skin tone. MBEH was shown to specifically target melanocytes and cause cell death via cell necrosis (18). As a selective effect of 8-DPAT on the melanocyte stem cell population was observed both *in vitro* and *ex vivo*, the systemic effects of long-term treatment were next examined in mice. These experiments offer an opportunity to examine the consequences of 8-DPAT treatment *in vivo*, as depigmentation can be successfully followed in mice in response to MBEH (18). The effects of 8-DPAT on C57BL/6 mouse pelage coloration were compared to that imposed by MBEH treatment (positive control). Hair was removed by Nair prior to applying MBEH or 8-DPAT in a racemic mixture to the ventral skin of mice every other day for 3 weeks. As shown in Fig. 5a and quantified as shown in Fig. 5b, MBEH caused marked depigmentation (mean change=79.1%, SD=19.6, N=5) when compared to pre-treatment control images

(mean change=-2.6%, SD=2.2, N=5) or 8-DPAT treatment (mean change=-2.8%, SD=3.0, N=5). Topical 8-DPAT application did not affect pigmentation of the mouse pelage within the 3 week treatment period.

MBEH and 8-DPAT target complementary cell populations in mice

MBEH is known to target mature melanocytes, whereas 8-DPAT appears to target primarily the melanocyte stem cell population in the skin. Results of in vitro and ex vivo experiments as shown above support selective melanocyte stem cell targeting by 8-DPAT. These complementary effects were further examined by immunohistochemical studies of the MBEH- and 8-DPAT-treated C57BL/6 mouse skin. Tissues were snap frozen after the 3 week treatment regimen described under Fig. 5, and the MBEH- and 8-DPAT treated tissues were compared to untreated skin (Fig. 6). Immunostaining for TRP1⁺ cells revealed a marked reduction of differentiated melanocytes in MBEH treated skin (measured by total area of each stained section, mean=7.9, SD=2.2, N=3). No difference was observed after treatment with 8-DPAT (mean=21.2, SD=5.4, N=3) versus vehicle (mean=29.5, SD=8.4, N=3), suggesting a negligible effect of this compound on mature melanocytes in vivo. Immunostaining for PAX3 expression, however, revealed markedly reduced staining in response to 8-DPAT (mean=12.0, SD=4.4, N=3) which was not observed in response to MBEH (mean=42.2, SD=8.9, N=3) or vehicle (mean=40.4, SD=3.1, N=3). Combining these outcomes, the data are congruent with a reduction in the number of melanocyte stem cells by 8-DPAT that has not (yet) affected the abundance of differentiated melanocytes. This dataset adds in vivo evidence for the proposed preferential targeting of melanocyte stem cells by 8-DPAT, and the complementary effects of these two compounds adds credence to the therapeutic potential of combined MBEH and 8-DPAT treatment for pigmentary disorders.

DISCUSSION

MBEH is an FDA approved topical agent for depigmentation therapy for patients with vitiligo. Its mechanism hinges on the presence of tyrosinase, the rate-limiting enzyme for melanogenesis, which converts MBEH to toxic quinones. Because melanogenic enzymes are expressed strictly by cells of melanocyte lineage, the cytotoxic effect of MBEH is confined to melanocytes (15,18,25). Resulting quinones are thought to react with melanosomal enzymes and cause haptenization of mature differentiation proteins, in particular tyrosinase. The immunogenicity of melanocyte and melanoma cells is increased as a result of antigen haptenization and cellular necrosis, which induces a melanoma-reactive CD8 T-cell immune response (26).

Our experiments again demonstrate that MBEH decreases viability of both melanocyte and, to a lesser degree, melanoma cell lines. Given this result, higher doses may be necessary for treatment of melanoma. Maximum achievable concentrations topically applied in vitiligo can produce undesirable side effects (27,28). Because MBEH is an organic phenol, liver and kidney toxicity are expected upon systemic application of drugs in this class (29).

Another limitation to using MBEH as a solitary drug against melanoma is its inability to reliably destroy melanocyte precursors. Indeed, repigmentation is commonly seen after cessation of the drug, and this most often occurs in a perifollicular pattern (27,28). It was

previously thought that poor penetration into the hair matrix caused the relative sparing of perifollicular melanocytes (30), however recent studies suggest that a stem cell subpopulation of melanocytes exists which are concentrated in the follicle and are resistant to these cytotoxic effects (27,30,31). This accounts for the same pattern of repigmentation upon exposure to UV light, which induces proliferation and differentiation of follicular melanocytes (30). Melanoma stem cells would therefore comprise a larger proportion of tumor after treatment with MBEH, as would be expected and was seen in the experiments presented here. Because this subpopulation of cells is likely primarily responsible for tumor expansion (6,32), MBEH treatment alone may ultimately be ineffective as a solitary treatment for melanoma.

Tumor development and metastasis is driven by stem cells. These cells can self-renew, differentiate, and can proliferate indefinitely (33), so effective therapies should target this subpopulation of cells which fuels melanoma growth. Metastatic melanoma cells cultured in growth medium suitable for human embryonic stem cells show different growth patterns and uniquely exhibit differentiation to multiple cell lineages (e.g., melanocytes, adipocytes, osteocytes) when compared to the same cells cultured in a standard medium (32). This supports that melanoma grows in concordance with the stem cell theory of cancer. In mouse models, very few stem cells are necessary to initiate tumor growth. In fact, experiments by Quintana et al have shown that in NOD/SCID mice, palpable tumor growth can be seen after xenotransplantation of just one human melanoma cell (34). Their potent ability to initiate and establish the heterogeneity of tumor makes stem cells an important target for future therapies.

To develop stem cell-targeting drugs, or to test the effectiveness of existing drugs on stem cell populations, it is essential to be able to identify stem cells by markers that are unique to them. These are often discovered by means of xenotransplantation of human melanoma cells into mouse models, and subsequent observation of stem cell behaviors such as tumor initiation or self-renewal. CD271 (nerve growth factor receptor) is a well-studied marker of melanoma precursor cells. Multiple studies have shown that CD271+ cells are multipotent, self-renew, establish full heterogeneity of tumor, and sustain longer-term growth compared to CD271-. They also tend to lack expression of TYR, MART-1, and MAGE proteins which accounts for their unresponsiveness to T cell therapies (9,19). CD34 is a marker commonly seen on hematopoietic cancer stem cells (35) and has more recently been shown to be present on tumor-initiating, self-renewing melanoma cells in mouse models (10). Additionally, pathologic analysis of malignant human melanoma biopsies has shown that CD34 is expressed (36,37). ABCB5 is a drug efflux transporter which regulates skin progenitor cell fusion and is a well-known marker of melanoma stem cells. CD20 and CD133 are additional markers of interest which have been shown to preferentially initiate tumor formation (38). Obviously, antibodies to any of these markers can be used to identify the stem cell population, yet most markers will be expressed by a more differentiated population of cells as well; for example, CD20 is commonly expressed by B cells. For that reason we used a combination of 3 stem cells markers to definitively identify the stem cell subpopulation among cultured cells.

Once the differences and commonalities between melanocytes and melanoma stem cells are clearly defined, a framework for targeted therapies can be established. Anatomically, melanocyte stem cells have a partial predilection for hair follicles and superficial nerve sheaths within the dermis, whereas mature melanocytes are dispersed within the epidermis (30,39,40). This anatomic relationship is evident in these experiments and in repigmentation patterns after cessation of MBEH treatment in vitiligo. Functionally, melanocyte stem cells lack the melanosomal machinery to produce melanin. These melanocyte differentiation proteins include tyrosinase, gp100, MART-1, TRP-1, and TRP-2, which bestow the ability to make pigment (41). This accounts for the underwhelming effect of MBEH reported here on melanocyte stem cells which retain a pluripotent state, with the ability to differentiate into multiple cell types and to initiate, establish the full heterogeneity of, and maintain the long-term growth of tumors. Immunologically, markers such as CD271 and ABCB5 are preferentially expressed in melanoma precursor cells, and xenotransplantation to mice of cells without these markers results in decreased tumor phenotype (38).

The Stem Cell Factor/Kit pathway is crucial pathway to regulating nearly all aspects of melanocyte homeostasis. Migration of melanocyte precursors and survival of both precursors and mature melanocytes is dependent on this intricate signaling pathway. There is a spatiotemporal relationship between perturbations in SCF/Kit and phenotype, as pigmentation is varied widely based on the precise timing of signal alteration (42,43). Interestingly, a unique subset of melanocyte stem cells which reside within hair follicles survives independent of SCF/Kit signaling (43). At the end of a hair cycle, melanocytes which die near the hair shaft are replenished by these Kit-independent survivors (42). SCF/Kit-targeted therapies such as imatinib are effective in treating gastrointestinal stromal tumors (GIST), which exhibit mutations in Kit. A subset of mutations of Kit in GIST also exists in some melanomas, and there has been success in decreasing proliferation of melanoma cells in-vitro using imatinib. Phase 2 trials of the drug in patients with c-Kit mutated metastatic melanoma have shown mixed results (46-48), but a minority of patients have achieved a long-lasting remission. Increased understanding of other cell signaling pathways involved in cancer development and proliferation such as BRAF/MEK and PI3K have proven instrumental in the development of several other drugs currently in phase II/III trials (44).

Experiments with zebrafish have elucidated much of melanocyte biology and the signaling pathways that mediate melanocyte development. Besides being a model for melanocyte migration and differentiation, zebrafish develop rapidly and have a sequenced genome, making them a suitable model for screening of small molecules. It was found that zebrafish embryos treated with 8-DPAT have significantly fewer migrated melanocytes. The results of a zebrafish screen show that 8-DPAT affects both melanocyte migration and survival via a Kit-dependent mechanism (14). 8-DPAT has been studied previously as a serotonin receptor agonist and reuptake inhibitor, suggesting that there may be an interface of serotonergic and melanogenic pathways. In this respect it is important to mention that 8-DPAT preferentially binds to the serotonin reuptake receptor 5-HT1A (49). Signaling via this receptor can, in turn, affect the survival and proliferation of neural stem cells, which may explain the cytotoxic effects of 8-DPAT apparent from the current studies (50).

Our data confirm that the Kit-dependent effects of 8-DPAT translate to other organisms beyond zebrafish and may have implications for the clinic. Application *in vitro* led to a marked decrease of stem cells in both melanocytes and melanoma cells cultures. Furthermore, application of 8-DPAT eliminates melanocyte stem cells from human skin explants. Topically applied to C57BL/6J mice, 8-DPAT failed to show a phenotypic effect on pelage pigmentation, but immunostaining for PAX3 revealed reduced populations of melanocyte precursors within hair follicles, where melanocyte stem cells are known to thrive. This clinically correlates to an ability to apply higher concentrations of 8-DPAT while avoiding pigmentary side effects that may be undesirable in melanoma. The data also support an ability of 8-DPAT to effectively penetrate the skin upon topical application, as previously suggested by transdermal delivery studies for closely related 5-DPAT (51).

We have shown that MBEH and 8-DPAT target complementary cell populations. The stem cells which remain after treatment with MBEH are thought to be responsible for the perifollicular repigmentation that occurs after drug cessation. Concordant with our data, MBEH exhibits a potent cytotoxic effect to mature melanocytes. Application of 8-DPAT in conjunction with MBEH may aid in decreasing the incidence and degree of repigmentation by destroying melanocyte stem cells that are otherwise resistant to other drugs. In melanoma, the minority stem cell population is thought to be responsible for tumor initiation, proliferation, and maintenance. These drugs together may form a formidable duo by eradicating the entire repertoire of aggressive melanoma, a therapeutic approach that may also prove effective in other diseases of aberrant pigmentation.

Acknowledgments

Support by NIH/NCI RO3 CA128068 to CLP is gratefully acknowledged.

ABBREVIATIONS

MBEH	monobenzyl ether of hydroquinone
8-DPAT	8-hydroxy-N,N-dipropyl-2-aminotetralin

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HIGHLIGHTS

- Monobenzyl ether of hydroquinone (MBEH) selectively targets mature melanocytes
- Skin treatment by MBEH induces rapid depigmentation, but viable stem cells remain
- 8-hydroxy-2-dipropylaminotetralin (8-DPAT) eliminates melanocyte/melanoma stem cells *in vitro*
- The 8-DPAT compound penetrates skin to reduce stem cell content *ex vivo* and *in vivo*
- 8-DPAT application may be effective in supporting treatment for vitiligo and melanoma

Figure 1. Physiologic changes in cultured melanocytes and melanoma cells in response to MBEH or 8-DPAT treatment

(a) Melanocyte (Mf12386 P1) and (b) melanoma (624.38) cell cultures were treated with vehicle alone, 250 μ M MBEH, or 500 μ M 8-DPAT for 24 hrs. The greatest physiologic changes were observed among primary melanocytes in response to MBEH.

Figure 2. Selective effects on cell viability are observed for MBEH treated melanocytes
MTT assays were performed on cell cultures after treatment with (a) MBEH and (b) 8-DPAT. Samples were plated in triplicate and treated for 24 hrs with 250μM or 500μM concentrations of MBEH or 8-DPAT. Cell lines tested include human melanoma cell lines 624.38, M14 and 888, B16 mouse melanoma cells, non-pigmented Jurkat immortalized human leukemic T cells (a negative control) and several foreskin-derived melanocyte cultures (Mf).

Figure 3. Stem cells are eliminated from melanocyte and melanoma cultures by 8-DPAT
(a) Melanocytes Mf13386 P1 and **(b)** 624.38 melanoma cells were treated for 24 hrs with or without 500µM of 8-DPAT for 24 hrs. Pax3/CD34/CD271 triple positive stem cells were significantly reduced by 30.5% and 35.6%, respectively. In **(c)**, fluorocytometric analysis of stem cell populations is shown for cultured 624.38 melanoma cells treated with MBEH alone, or with a combination of MBEH and 8-DPAT. Stem cells are enriched among MBEH treated cells, whereas the population is markedly reduced when 8-DPAT (500 µM) is also added to 250 µM MBEH treated cells. Stem cells were identified by fluorocytometric analysis of the triple positive stem cell population among viable cells, and numbers were recalibrated for cell viability to determine the reduction in stem cells in response to treatment.

Figure 4. Melanocyte stem cells are selectively targeted by topical application of 8-DPAT *ex vivo* Human skin explants were treated with 100 μ l of 250 μ M MBEH, 500 μ M 8-DPAT or vehicle alone and maintained for 48 hrs before preparing cryosections to detect co-expression of Pax3 (green arrows) and CD271 (red arrows) among nucleated cells. On average, approximately 40% loss of double positive (stem) cells (yellow arrows) was estimated in samples from 3 donors after treatment with 8-DPAT; no change was observed in response to MBEH (not shown).

Figure 5. Topical treatment by MBEH, but not 8-DPAT induces depigmentation in C57BL/6 mice

C57BL/6 mice (5 per group) were ventrally treated with 100ul of vehicle, MBEH, or 8-DPAT applied to Naired skin every other day for 3 weeks. After treatment, the mice were imaged weekly using a flatbed scanner. **(a)** Representative images of the scanned mice 3 weeks after the end of treatment. **(b)** Depigmentation was calculated over time. MBEH caused significant depigmentation over time (mean change=87.8%, SD=10.5) versus vehicle (-2.6%, SD=2.2) or 8-DPAT (-2.8%, SD=3.0).

Figure 6. MBEH and 8-DPAT target different subpopulations of melanocytic cells upon topical application

C57BL/6 mice treated with 100 μ l of 225 mM MBEH, 450 mM 8-DPAT or vehicle alone every other day for 3 weeks before tissues were snap frozen for immunohistochemistry. **(a)** Showing untreated, MBEH treated and 8-DPAT treated mouse skin from left to right, immunostaining reveals a loss of TRP-1⁺ melanocytes in response to MBEH (mean=7.9, SD=2.2), whereas this differentiated melanocytes population was not affected by 8-DPAT treatment (mean=21.2, SD=5.4) or vehicle (mean=29.5, SD=8.4). **(b)** In response to 8-DPAT treatment, the abundance of Pax3⁺ cells (which includes the melanocyte stem cell population), is reduced (mean=12.0, SD=4.4) compared to MBEH (mean=42.2, SD=8.9) and vehicle (mean=40.4, SD=3.1).