Monobenzyl Ether of Hydroquinone and 4-Tertiary Butyl Phenol Activate Markedly Different Physiological Responses in Melanocytes: Relevance to Skin Depigmentation

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Monobenzyl ether of hydroquinone (MBEH) is a Food and Drug Administration approved drug used for depigmentation therapy of advanced vitiligo. Here, the working mechanism of MBEH is explored in comparison to 4-tertiary butyl phenol (4-TBP), a known causative agent for occupational vitiligo mediating apoptotic melanocytic death. Cytotoxic experiments reveal that similar to 4-TBP, MBEH induces specific melanocyte death. To compare death pathways initiated by 4-TBP and MBEH, classical apoptotic hallmarks were evaluated in treated melanocytes. MBEH induced cell death without activating the caspase cascade or DNA fragmentation, showing that the death pathway is non-apoptotic. Release of High Mobility Group Box-1 protein by MBEH-treated melanocytes and ultrastructural features further confirmed a necrotic death pathway mediated by MBEH. A negative correlation between MBEH-induced cell death and cellular melanin content supports a cytoprotective role for melanin. Moreover, MBEH exposure upregulated the levels of melanogenic enzymes in cultured melanocytes and skin explants, whereas 4-TBP reduced the expression of the same. In summary, exposure to MBEH or 4-TBP has profoundly different consequences for melanocyte physiology and activates different death pathways. As the mode of cell death defines the nature of the immune response that follows, these findings help to explain the relative efficacy of these agents in mediating depigmentation.

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INTRODUCTION

Vitiligo, a chronic pigmentary disorder, results from a T-cell mediated autoimmune response toward melanocytes (Le Poole and Luiten, 2008). Patients with advanced vitiligo can opt to biochemically remove remaining pigmentation (Lim and Camile, 2007). For depigmentation therapy, new methods are gradually becoming available including the use of 4-methoxy phenol combined with Q-switched ruby laser or cryotherapy (Lotti *et al.*, 2008). However, monobenzyl ether of hydroquinone (MBEH) remains the only drug that the Food and Drug Administration approved for depigmentation therapy within the United States (Bolognia *et al.*, 2001). The working mechanism of MBEH remains unexplored, although MBEH is believed to remove melanocytes from the skin, as it generally mediates permanent depigmentation (Bolognia *et al.*, 2001). However, in some patients, repigmentation has been observed after treatment (Oakley, 1996).

The depigmentation process that follows exposure to phenolic agent 4-tertiary butyl phenol (4-TBP) is better understood. Occupational vitiligo in some individuals working in the rubber and tannery industries has been attributed to 4-TBP (Boissy and Manga, 2004). *In vitro* studies have shown that 4-TBP is specifically cytotoxic to melanocytes. Though 4-TBP is a tyrosine analog that binds the catalytic site of the tyrosinase enzyme and acts as a competitive inhibitor of tyrosinase (Yang and Boissy, 1999), the cytotoxic effects were found to be independent of tyrosinase activity (Yang and Boissy, 1999; Yang *et al.*, 2000). The latter studies revealed that sensitivity of melanocytes to 4-TBP instead correlates with the level of tyrosinase-related protein 1 (TRP-1) expressed by melanocytes (Manga *et al.*, 2006). Normal melanocytes were shown to downregulate the levels of microphthalmia-associated transcription factor and TRP-1 in

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Abbreviations: 4-TBP, 4-Tertiary butyl phenol; HMGB1, High Mobility Group Box-1 protein; MART-1, melanoma antigen recognized by T cells-1; MBEH, mono benzyl ether of hydroquinone; PARP, poly (ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; TRP, tyrosinase-related protein

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response to 4-TBP as a protective mechanism (Manga *et al.*, 2006). Finally, 4-TBP is shown to activate apoptosis in melanocytes (Yang *et al.*, 2000).

The two main categories of cell death are apoptosis and necrosis. Apoptosis is a well defined and programmed cell death with classical hallmarks such as membrane blebbing, nuclear fragmentation, activation of the caspase cascade, and changes in mitochondrial permeability (Krysko *et al.*, 2008). As an early signal, cells undergoing apoptosis display surface expression of phosphatidyl serine and are opsonized for phagocytosis by phosphatidyl serine receptor expressing macrophages (Fadok *et al.*, 1992, 1998). Alternatively, necrosis is an unregulated form of cell death characterized by cytoplasmic swelling, and disintegration of the plasma membrane and cellular organelles (Krysko *et al.*, 2008). Necrotic cells stimulate an inflammatory response by releasing immune stimulatory agents like DNA-binding High Mobility Group Box-1 protein (HMGB1), heat shock proteins, DNA and RNA (Scaffidi *et al.*, 2002; Krysko *et al.*, 2006), and necrosis is therefore considered a strong inducer of immune responses (Krysko *et al.*, 2006).

The current study is focused on understanding whether MBEH intersects with the melanogenic pathway and mediates selective cytotoxicity to melanocytes by *in vitro* cytotoxicity assays using melanocytes, fibroblasts, and keratinocytes isolated from human skin. The appearance of apoptotic hallmarks in response to chemical exposure was assessed by FACS analysis of surface phosphatidyl serine expression as an early apoptotic marker. Activation of the caspase cascade was assessed by western blotting and fluorimetric assays to identify products of caspase-3 and poly (ADP-ribose) polymerase (PARP) cleavage. Possible alternative modes of cell death were followed by electron microscopic comparison of cultured cells exposed to 4-TBP or MBEH followed by analysis of HMGB1 release in supernatants of treated cells. Studies were expanded to include a model more closely mimicking human skin; DNA fragmentation was analyzed by TUNEL staining of organotypic skin cultures. Further, the potential involvement of melanogenic enzymes in depigmentation was assessed by correlating sensitivity to MBEH with basal expression levels of melanogenic enzymes and melanin content among melanocyte monocultures measured by FACS analysis and a standard quantitative melanin assay. Changes in the expression levels of melanosomal markers on treatment with 4-TBP or MBEH were identified by FACS analysis of cultured cells, and confirmed by immunohistochemistry of cultured skin explants. Besides a further understanding of the consequences of topical exposure to bleaching agents *per se*, the current investigations shed light on the potential of these agents to achieve complete and permanent depigmentation of the skin in vitiligo patients.

RESULTS

Cytotoxicity quantification

The viability of cultured human primary melanocytes, fibroblasts, and keratinocytes was determined through MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays after treating with 250, 500, and 900 μM of 4-TBP or MBEH for 24 hours. In Figure 1, data are shown for a representative of two experiments performed. Vehicle treatment served as a control for all cell types. In Figure 1a, the viability of melanocytes was reduced by 26 and 67% in response with 250 and 500 μM of 4-TBP, respectively. Upon 250 and 500 μM concentrations of MBEH treatment, the viability of melanocytes was reduced by 43 and 77%, respectively, as shown in Figure 1b, indicating an increased potency of MBEH in comparison with 4-TBP. Surprisingly, fibroblasts and melanocytes were equally sensitive to both treatments, yet keratinocytes were significantly more resistant to both 250 or 500 μM concentrations. The viability of keratinocytes remained unchanged upon 250 μM exposure, whereas at 500 μM the viability was reduced only by 21 and 31% on 4-TBP and MBEH treatment, respectively. Thus, both bleaching phenols are selectively cytotoxic to melanocytes among epidermal cells. Finally, at the highest concentration, viability was not observed in any cell type for either treatment.

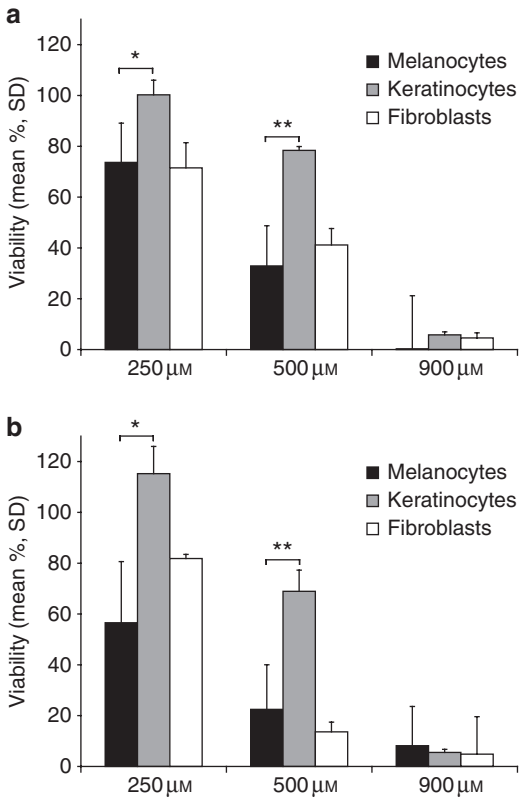


Figure 1. Cytotoxicity of monobenzyl ether of hydroquinone (MBEH) and 4-tertiary butyl phenol (4-TBP) toward cutaneous cells. Epidermal foreskin-derived melanocytes Mf0814 P5, Mf0932 P2, Mf0883 P3, and Mf0929 P3 with respective melanin contents of 55.0, 10.7, 8.1, and 28.4 pg per cell, fibroblasts (Ff0201 P4) and keratinocytes (Kf0180 P14) were treated with 250, 500 and 900 μM of 4-TBP (**a**) or MBEH (**b**) for 24 hours. The percent viability was calculated through MTT assays. Mean and SD were calculated and statistical significance was evaluated by Student's *t*-test. $*P<0.05$ and $**P<0.01$ for melanocyte viability in comparison with keratinocytes after 250 and 500 μM treatment of 4-TBP and MBEH treatment.

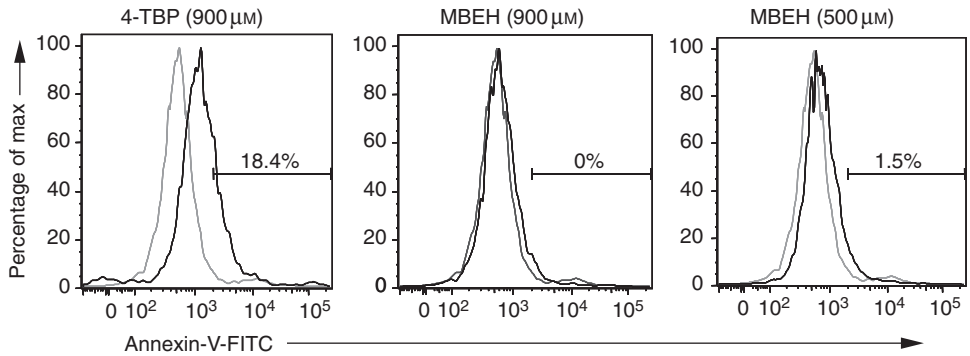


Figure 2. Annexin-V staining of monobenzyl ether of hydroquinone (MBEH)- and 4-tertiary butyl phenol (4-TBP)-treated cells. Melanocytes were treated with 500 or 900 μM of 4-TBP or MBEH (solid line) or vehicle alone (gray line) for 1 hour. FACS analysis of annexin-V staining, gating out PI-stained (dead) cells, showed that 4-TBP but not MBEH-induced apoptotic cell death.

Analysis of annexin-V staining

Melanocytes treated with 4-TBP or MBEH (500 or 900 μM for 1 hour) were stained with FITC-labeled antibody to annexin-V along with propidium iodide. Propidium iodide-positive cells were gated out and the percentage of apoptosis was estimated in live cells. At 900 μM of 4-TBP, melanocytes revealed a substantial apoptotic population (18.4%) as shown in Figure 2. As melanocytes are more sensitive to MBEH than to 4-TBP as shown in Figure 1, data are included for 1-hour treatment with both 900 and 500 μM of MBEH to ensure that apoptosis is not induced at a lower concentration of the drug. Figure 2 further confirms that no apoptosis was observed on treatment with MBEH. Cells treated with vehicle alone served as controls for both treatments.

Caspase-3 and PARP detection

Cleavage of caspase-3 is a critical indicator of the apoptotic cascade. To quantify the enzymatic activity of caspase-3, cell extracts pre-treated with either agent for 1 hour were incubated with a synthetic caspase-3 peptide substrate that emits fluorescence on cleavage. In a representative experiment shown in Figure 3a, MBEH treatment did not impact the level of caspase-3 activity in comparison with vehicle treatment at either concentration used. However, melanocytes treated with 4-TBP significantly increased caspase-3 activity in response to the 900 μM concentration ($P<0.05$). Western blot analysis shown in Figure 3b further confirmed that MBEH treatment did not induce caspase-3 cleavage, whereas 4-TBP treatment induced cleavage of pro-caspase into active caspase. Cleavage of PARP, a downstream target of caspase-3, was similarly evaluated by western blotting. As expected, PARP cleavage was observed in response to treatment with 900 μM of 4-TBP, but not following MBEH treatment (Figure 3c).

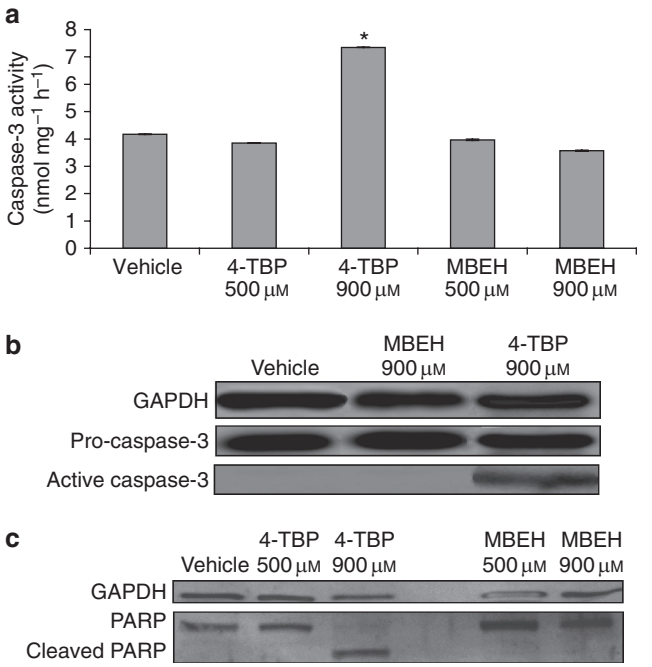
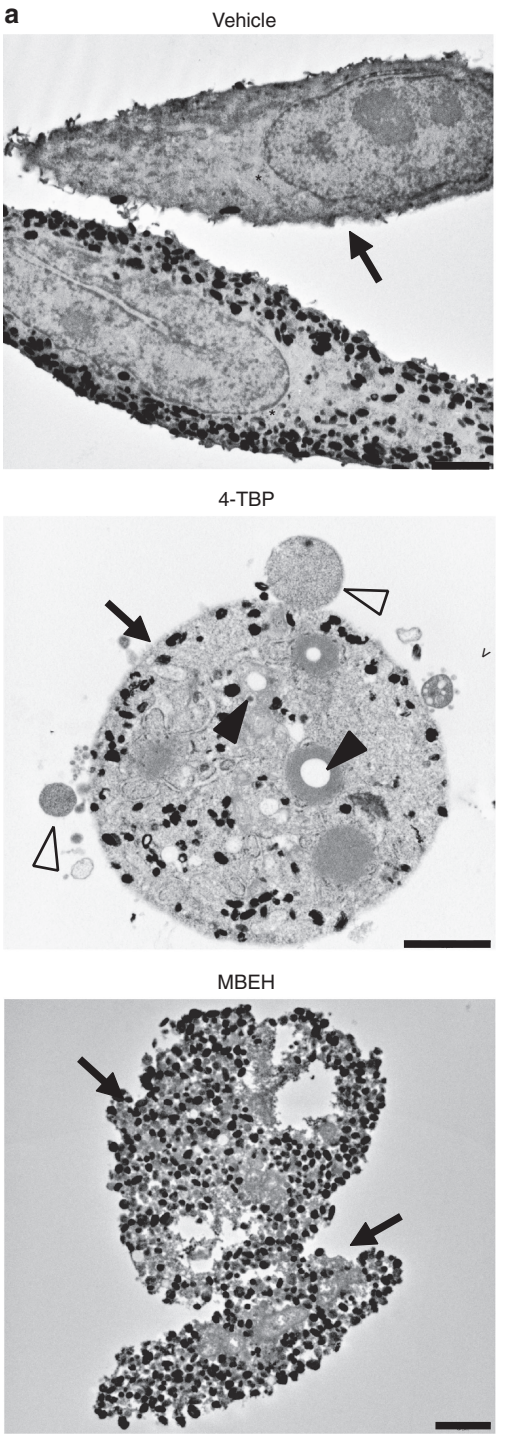


Figure 3. Caspase-3 and poly (ADP-ribose) polymerase (PARP) in MBEH and 4-TBP-treated cells. Melanocytes were treated with 500 or 900 μM of 4-TBP or MBEH for 1 hour and analyzed for (**a**) caspase-3 activity, (**b**) cleavage of caspase-3, and (**c**) PARP cleavage. (**a**) Caspase-3 activity (nanomoles per milligram per hour) was quantified based on the amount of fluorescence emitted on cleavage of the substrate Ac-DEVD-AFC. Statistical significance at $*P<0.05$ was observed for caspase-3 activity after 900 μM treatment of 4-TBP over vehicle treatment. (**b**) Cleavage of caspase-3 identified through western blotting revealed no caspase-3 cleavage after MBEH treatment, whereas 4-TBP treatment does induce cleavage of pro-caspase (32 kD) into active caspase (11 kD). (**c**) Treatment with 4-TBP treatment induced cleavage of PARP (116 kD) into inactive PARP (89 kD), whereas MBEH treatment did not induce PARP cleavage. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Analysis of necrotic changes

Ultrastructural changes in response to 4-TBP and MBEH exposure were examined by electron microscopy. Physiological changes in treated cells were most readily demonstrable at the 1 mM concentration for 1 hour, as shown in representative images of individual cells in Figure 4a. An electron micrograph of intact cells is shown for vehicle treatment alone. Exposure to 4-TBP produced defined apoptotic features including formation of apoptotic bodies, cytoplasmic vaculorization, blebbing of the plasma membrane, and a condensed nucleus while maintaining plasma membrane integrity. MBEH-treated cells revealed typical necrotic



features such as disruption of the plasma membrane and release of cellular contents. Even at 125-μM treatment for 4 hours, similar ultrastructural features could be observed in response to MBEH, but not to 4-TBP (not shown). Moreover, the release of HMGB1 in the supernatants of melanocytes treated with 900 μM of 4-TBP or MBEH for 1 hour was analyzed through western blotting. As expected, HMGB1 was released on MBEH treatment alone, further confirming the necrotic pathway (Figure 4b).

Confocal analysis of DNA fragmentation in exposed skin

DNA fragmentation in treated explants was analyzed by TUNEL staining. Human skin explants of 6 mm in diameter were treated with 5 μl of 250 mM of 4-TBP or MBEH for 1 day. Control explants were treated with vehicle alone. Cryosections were immunostained with antigen-presenting cell-labeled anti-MART-1 (melanoma antigen recognized by T cells-1), incorporating FITC-labeled nucleotides with the Klenow fragment of DNA polymerase to free ends of fragmented DNA. Sections were counterstained with 4′, 6-diamidino-2-phenylindole to identify cell nuclei. Stained sections were analyzed by confocal microscopy (Figure 5). Similar to control samples, MBEH-treated explants showed focal DNA fragmentation in the granular layer of the epidermis and in the dermis, whereas no apoptotic signal was observed throughout the basal layer where melanocytes are situated. Apoptotic melanocytes were observed in 4-TBP-treated skin with colocalization of TUNEL (in green) and MART-1 staining (red). 4-TBP treatment induced DNA fragmentation in 80% of basal melanocytes within skin explants as opposed to MBEH treatment where not a single apoptotic melanocyte was observed. Note that at the high concentration of either agent used, keratinocyte apoptosis was observed within 4-TBP-treated explants only, further emphasizing the contrast between both agents.

Role of pigmentation in MBEH-mediated death

A role for melanin in the toxicity induced by MBEH was identified by correlating melanocyte viability after MBEH exposure to melanin levels observed in cultured melanocytes. In Figure 6a, a positive correlation between cellular melanin content and viability after MBEH treatment was observed, with a correlation coefficient of 0.6. By contrast, melanin

Figure 4. Analysis of necrotic changes. (**a**) Morphological changes in melanocytes treated with 1 mM 4-tertiary butyl phenol (4-TBP) or monobenzyl ether of hydroquinone (MBEH) or vehicle alone for 1 hour were evaluated by electron microscopy. Vehicle-treated cells show intact plasma membranes (arrows) and nuclear membranes (asterisks). 4-TBP treatment induced apoptotic changes, including cytoplasmic vacuolarization (filled arrowheads), plasma membrane blebbing (open arrow heads), and formation of apoptotic bodies (carrot symbol). MBEH treatment induced necrotic changes consisting of plasma membrane (arrows) and cytoplasmic disintegration. Bars = 2 μm. (**b**) Western blot analysis of melanocyte (Mf0930 P2) supernatant to detect High Mobility Group Box-1 protein (HMGB1) after treatment with 900 μM of 4-TBP or MBEH for 1 hour. Release of HMGB1 was identified after MBEH treatment alone. Serum albumin bands observed in the respective supernatants were shown to demonstrate equal loading.

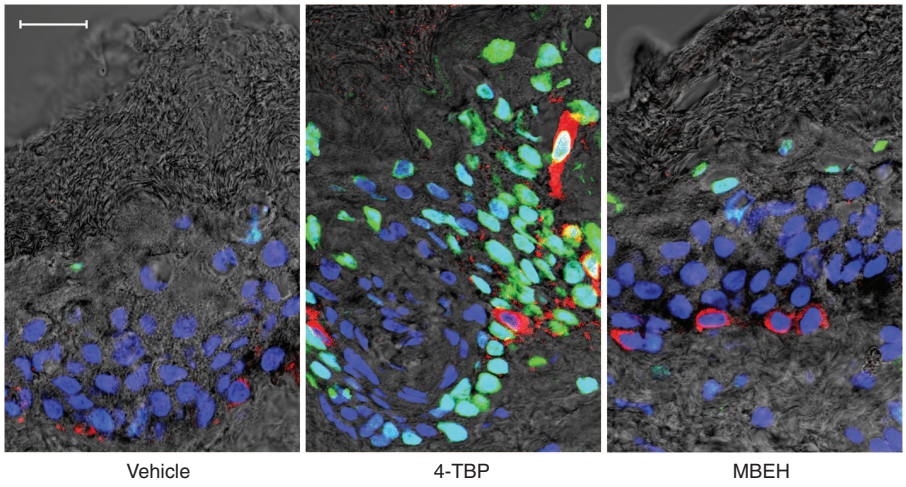


Figure 5. DNA fragmentation within monobenzyl ether of hydroquinone (MBEH)- or 4-tertiary butyl phenol (4-TBP)-treated skin. Apoptosis was shown in confocal microscopic images of explant cultures exposed to 5 µl of 250 mM 4-TBP or MBEH for 24 hours. Vehicle treatment alone served as control. Melanocytes immunostained with antibodies to MART-1 were shown in red and DNA fragmentation was represented in green. Nuclear counterstaining by DAPI was observed in blue. Colocalization of all three colors is visible in white. Vehicle- and MBEH-treated skin sections lacked DNA fragmentation in the basal epithelial layer, whereas 4-TBP treatment showed melanocytes expressing DNA fragmentation. Bar = 20 µm.

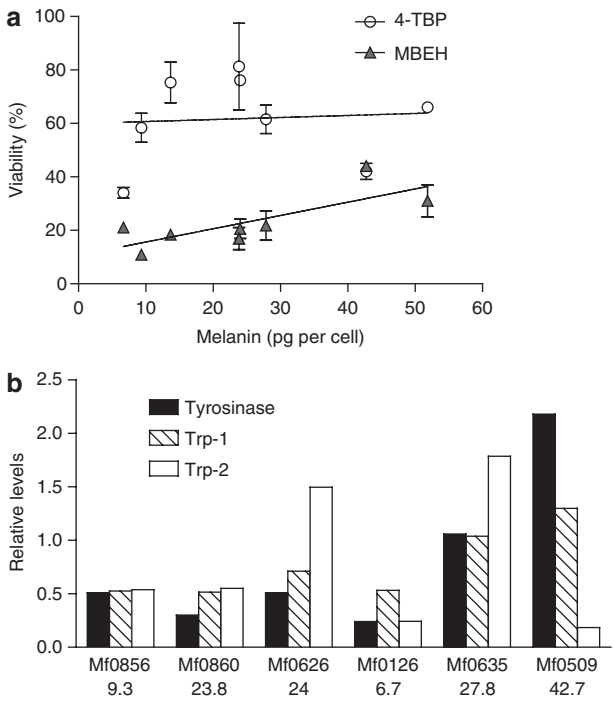


Figure 6. Role of pigmentation in monobenzyl ether of hydroquinone (MBEH)-mediated death. (**a**) Melanocytes were treated with 500 µM of 4-tertiary butyl phenol (4-TBP) or MBEH for 24 hours and cell viability was quantified through MTT assays. Melanocyte viability was correlated with baseline cellular melanin content. (**b**) The baseline expression levels of TRP-1, tyrosinase, and TRP-2 were quantified by FACS analysis. Expression levels of melanogenic enzymes reported for individual melanocyte cultures were arranged according to increasing melanocyte viability on MBEH exposure from left to right. The melanin content of each melanocyte culture was reported in picogram per cell underneath the culture name.

content did not correlate with cell death mediated by 4-TBP. In Figure 6b, expression levels of melanogenic enzymes tyrosinase, TRP-1, and TRP-2 was shown for six melanocyte cultures arranged from left to right according to increasing viability in presence of MBEH. Indeed, sensitivity to MBEH was not specifically associated with the levels of expression of any of these melanogenic enzymes. Interestingly, whereas the expression levels of tyrosinase and TRP-1 vary with melanin content, no such association was shown for TRP-2, suggesting that TRP-2 expression is of less importance for melanization. Taken together, the data support a protective role of melanin in MBEH-induced cell death.

Melanosomal marker analysis through intracellular staining

Relative changes in the expression of melanosomal proteins were identified in melanocytes pre-treated with 4-TBP or MBEH *versus* treatment with vehicle alone. As shown in Figure 7, 4-TBP-treated melanocytes expressed levels of TRP-1, tyrosinase and, TRP-2 reduced by 37, 20, and 23%, respectively. MBEH-treated melanocytes displayed an opposite trend with the levels of TRP-1, tyrosinase, and TRP-2 increased by 13, 67, and 81% over vehicle-treated cells, respectively.

Melanosomal marker analysis in *ex vivo* organotypic cultures

Changes in the expression of melanosomal marker proteins were further followed in an *ex vivo* environment through organotypic culture of human skin treated daily with 5 µl of 4-TBP or MBEH. Analysis of immunostained sections confirmed observations reported for FACS analysis of cultured cells described above. After 24 hours, treatment with 250 mM of 4-TBP markedly downregulated the expression of gp100, TRP-1, tyrosinase, MART-1, and TRP-2, whereas slightly increased expression of the same markers was observed after

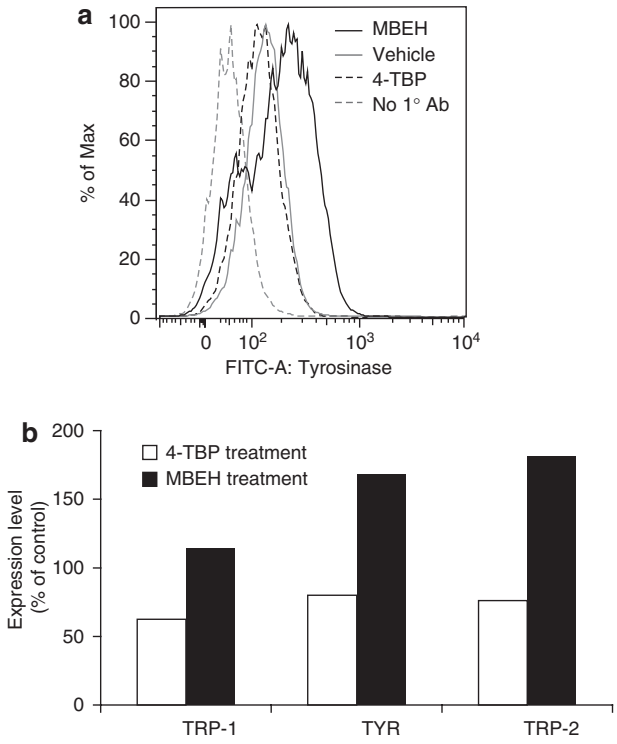


Figure 7. Melanosomal marker analysis in cultured melanocytes. Melanocytes (Mf0639 P6) were treated with 250 μM of 4-TBP or monobenzyl ether of hydroquinone (MBEH) for 72 hours. The cells were stained for expression of TRP-1, TRP-2 and tyrosinase, and the expression levels were quantified by FACS analysis. (**a**) Histogram showing representative FACS staining for tyrosinase and (**b**) bar graph indicating relative expression levels of tyrosinase-related protein-1 (TRP-1), tyrosinase (TYR), and tyrosinase-related protein-2 (TRP-2) compared with vehicle-treated cells.

MBEH treatment (Figure 8). It should also be noted that 4-TBP treatment downregulated the levels of anti-apoptotic factor Bcl-2, whereas no appreciable change was observed in response to MBEH. Images shown are from a representative of three experiments performed.

DISCUSSION

MBEH (10–20%) is used in dermatology clinics within the United States for depigmentation treatment of patients with advanced vitiligo (Bolognia *et al.*, 2001). The current studies report on the underlying mechanism by which MBEH mediates human skin depigmentation, which, to our knowledge, has not previously been reported. Viability assays showed that similar to 4-TBP, MBEH induces specific toxicity toward melanocytes, as previously suggested by studies in mice (Quevedo *et al.*, 1990; Yang and Boissy, 1999). At the same time, fibroblast and melanocytes are equally sensitive to both the agents, suggesting that neither agent should be used for systemic administration, thus limiting their use to topical applications. Previous studies have reported that the mechanism of melanocyte destruction by 4-TBP is through apoptosis (Yang *et al.*, 2000). MBEH-induced cell death was therefore expected to follow a similar death pathway. Surprisingly, MBEH treatment of cultured melanocytes induced unique morphological changes when compared with 4-TBP treatment. Although 4-TBP treatment induced perinuclear vacuolarization and membrane ruffling, MBEH treatment induced disintegration of cellular membrane and release of cellular contents as observed by light microscopy (data not shown). This suggested that the mechanism of death initiated by MBEH may be different from that observed in response to 4-TBP. Further studies confirmed that MBEH-induced cytotoxicity lacked classical apoptotic hallmarks such as caspase-3 activity, cleavage of caspase-3 or PARP proteins, exposure of phosphatidyl serine, and DNA fragmentation. Further, a downregulation of Bcl-2 expression in the basal epidermal layer of treated explant cultures was observed on 4-TBP treatment, but not in response to MBEH. Electron micrographs confirmed the presence of necrotic changes including disruption of the plasma membrane and the nuclear membrane. Furthermore, release of HMGB1 was observed after MBEH treatment alone, further emphasizing the same fact. Previous studies have shown that active PARP is required for the release of HMGB1 (Ditsworth *et al.*, 2007) . This explains the lack of HMGB1 release on 4-TBP treatment, as 4-TBP induces cleavage and inactivation of PARP.

During programmed apoptotic cell death, inflammation is a rare event, although macrophage influx is required for a rapid clearance of apoptotic cells (Chung *et al.*, 2000; Krysko *et al.*, 2006). If apoptotic cells are not phagocytized immediately, dying cells are cleared through a necrosis-like mechanism referred to as secondary necrosis. Though secondary necrosis is characterized by certain necrosis-like changes, nuclear fragmentation and chromatin condensation are also observed (Silva *et al.*, 2008). MBEH-mediated cytotoxicity does not induce regulated nuclear fragmentation as seen by TUNEL staining, thus ruling out secondary necrosis as the mechanism of cell death. Taking into account the structural, morphological, and biochemical changes observed, it was clearly shown that MBEH induces necrotic death in human melanocytes.

Correlative studies between melanin content and melanocyte sensitivity on MBEH exposure indicate that melanin acts as a protective agent against MBEH-induced cell death. This implies that people with a darker complexion may benefit less from MBEH depigmentation treatment. This is countered by the fact that recent studies implicate tyrosinase as the enzyme mediating depigmentation in response to MBEH (Westerhof and d'Ischia 2007; van den Boorn *et al.*, 2008). In this regard, MBEH seems to be converted into a quinone product on interaction with tyrosinase (van den Boorn *et al.*, 2008). The accumulation of such quinone products may induce toxicity selectively in melanocytes (Riley, 1970). Alternatively, the possibility should be considered that MBEH preferentially enters melanocytes as previously shown for 4-hydroxy anisole in melanocyte–keratinocyte co-cultures (Riley, 1970). In either case the melanosome likely hosts the depigmenting compound, as further supported by the protective effect of melanin against MBEH-induced cell death.

The upregulation of melanosomal markers through intracellular and immunohistochemistry on MBEH treatment may in part explain the repigmentation observed in some

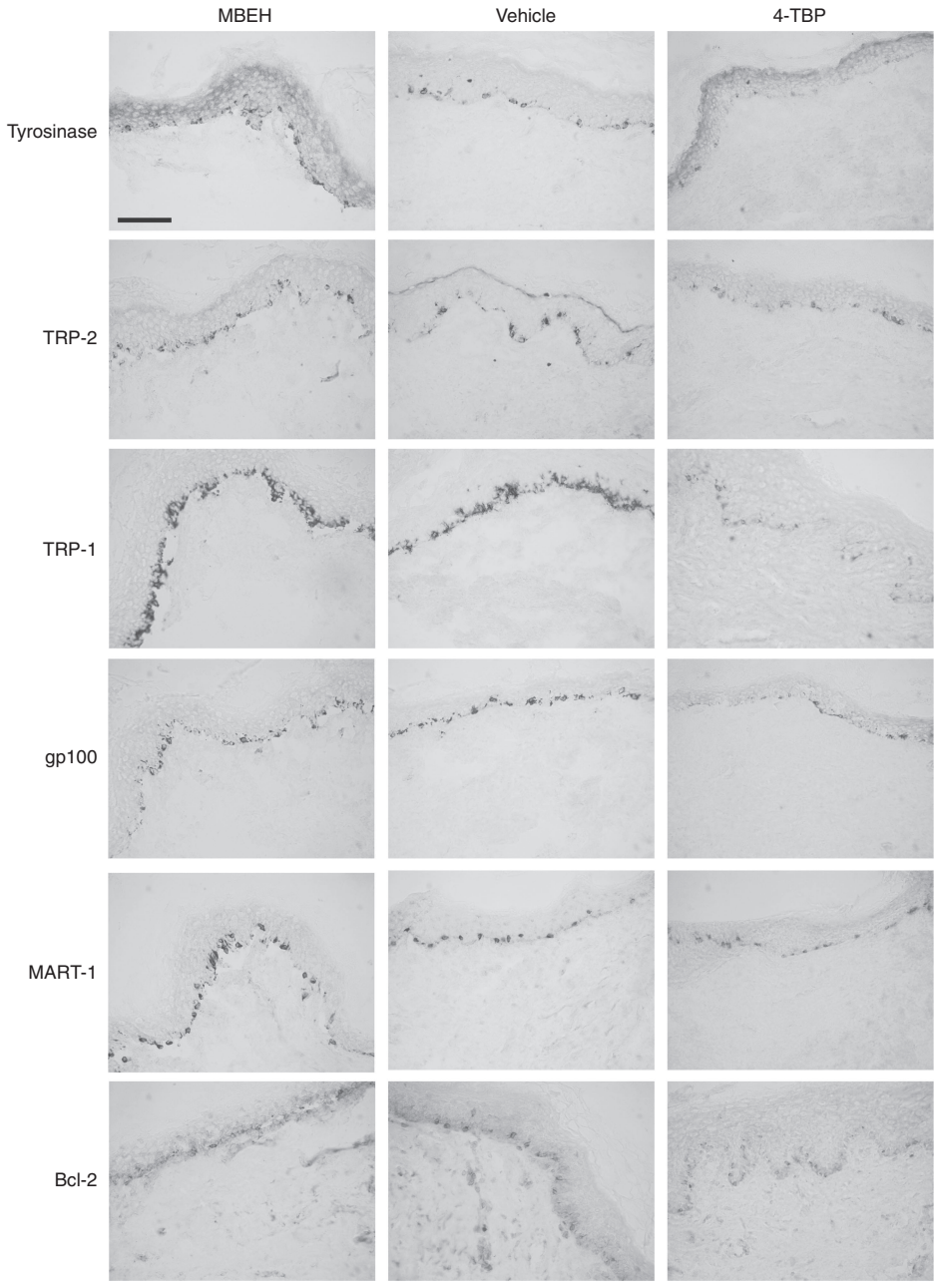


Figure 8. Melanosomal marker analysis in *ex vivo* organotypic cultures. Explant skin cultures were treated with 250 mM of 4-tertiary butyl phenol (4-TBP) or monobenzyl ether of hydroquinone (MBEH) for 24 hours. Vehicle treatments alone served as controls. The vertical columns represent respective treatments and the immunohistochemistry images representing expression levels of tyrosinase, TRP-2, TRP-1, gp100, MART-1, and Bcl-2 are shown in horizontal columns. Sections representing 4-TBP treatment revealed markedly downregulated levels of melanosomal markers as well as of Bcl-2, whereas MBEH treatment slightly upregulated the levels of expression of all markers studied by melanocytes in the basal layer of the epidermis. Bar = 75 μm.

patients after depigmentation therapy (Bolognia *et al.*, 2001), although a repopulation of depigmented skin by melanocytes is likely required for such repigmentation to occur. Therefore, it is postulated that a balance exists between the protective effects of melanin and the toxic effects of quinone accumulation. Cell death occurs if quinone accumulation prevails. This explains the lack of a direct association between the sensitivity to MBEH and the basal levels of tyrosinase, TRP-1, or TRP-2, as the presence of melanin overshadows the toxic effects of quinone products generated on interaction with increasing levels of melanosomal enzymes. This observation contrasts with the observed decrease in TRP-1 levels associated with increased viability on 4-TBP exposure (Manga *et al.*, 2006). Overall, chemical exposure was accompanied by opposing consequences for the expression of melanosomal enzymes both *in vitro* and *ex vitro*. In conclusion, different mechanisms are involved in chemical depigmentation of the skin by 4-TBP and MBEH. This leaves

the intriguing question why one agent induces apoptosis and the other necrosis of the same target cells. The explanation may in part be found in the different side chains to either phenolic agent and in the resulting differences in molecular structure potentially imposing differential reactivity towards melanocytes.

Both 4-TBP and MBEH are specifically cytotoxic to melanocytes on topical application with vastly different physiological consequences, favoring the application of MBEH in a treatment setting. The data imply that MBEH is more likely to invoke an inflammatory response and induce autoreactivity to melanosomal antigens, which may explain distant depigmentation away from the application site observed for this compound (Bolognia *et al.*, 2001) It may be possible to identify previously unreported molecular derivatives or combinatory treatments providing more rapid depigmentation of the skin. The current studies provide an incentive for studies to unravel the possible impact of either agent on immunological responses to dying melanocytes, which can impact on the permanent features of depigmentation.

MATERIALS AND METHODS

Cell culture

Cultures of normal human melanocytes, keratinocytes, and fibroblasts were established from otherwise discarded foreskin tissue obtained after routine circumcision. All studies performed with human tissue were carried out in adherence with the Declaration of Helsinki Principles and were approved by the Institutional Review Board of the institution where circumcisions were performed. Patient consent was not necessary. Dermo-epidermal separation was performed by incubation with 0.5 mg ml^{-1} thermolysin (Sigma, St Louis, MO). Epithelial cells were further separated by incubation in 0.1% trypsin. Cells or dermal tissue were plated in keratinocyte, melanocyte, or fibroblast growth media.

Normal human melanocytes were cultured in Ham's F-12 medium (Media-Tech, Herndon, VA) with 2 mM Glutamine (Invitrogen, Carlsbad, CA), 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 100 μg ml^{-1} amphotericin (Invitrogen), 0.1 mM isobutylmethylxanthine (Sigma), 10 ng ml^{-1} 12-O-tetradecanoylphorbol-13-acetate (Sigma), and 1% Ultroser G (Pall Biosepra, Cergy, France).

Normal human dermal fibroblasts were cultured in DMEM (Media-Tech) with 10% heat-inactivated fetal bovine serum (Gemini Bio-products, West Sacramento, CA), 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 100 μg ml^{-1} amphotericin (Media-Tech).

Normal human keratinocytes were cultured in growth medium with M154 basal medium (Cascade Biologicals, Portland, OR), supplemented with human keratinocyte growth supplements (Cascade Biologicals) with 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 100 μg ml^{-1} amphotericin (Invitrogen), and immortalized with HPV16 E6 and E7 retroviral constructs as described previously to allow prolonged passaging (Halbert *et al.*, 1991).

Organotypic culture

Organotypic culture was performed as described previously (Le Poole *et al.*, 1994) . In brief, 6-mm biopsies were punched from otherwise discarded foreskin tissue obtained after routine circumcision from University of Chicago. The explants were cultured in DMEM containing 10% inactivated normal human serum at the air–liquid interphase in 0.4 μm transwell plates with epidermal side facing up (Corning, Teterboro, NJ). After treatment with 5 μl of 250 mM of 4-TBP or MBEH for 24 hours, explants were embedded in optimal cutting temperature compound (OCT) (Sakura Finetek USA, Torrance, CA) and snap frozen for future sectioning. All studies performed with human tissue were carried out in adherence with the Declaration of Helsinki Principles and were approved by the Institutional Review Board of the institution where circumcisions were performed.

Cytotoxicity assay

Cell viability was measured by MTT assays (Bioassay system, Hayward, CA) according to manufacturer's instructions. In brief, 20,000 cells per well were plated in triplicate wells of a 96-well plate to attach overnight. Cells were either treated with vehicle alone or with 250 or 500 μM of 4-TBP or MBEH for 24 hours. 4-TBP (Sigma) was prepared as a stock solution of 250 mM in 70% ethanol. MBEH (Sigma) was dissolved in 20% dimethyl sulfoxide and mixed with 70% ethanol for a stock concentration of 250 mM. The use of a vehicle control refers to the use of 20% dimethyl sulfoxide in 70% ethanol. MTT reagent (tetrazole) was added to the cells and incubated in a 37°C humidified chamber for 4 hours. Tetrazole is converted to formazan in the mitochondria of living cells. The formazan crystals formed were dissolved in solubilization buffer and the absorbance was read in a spectrophotometer at a wavelength of 562 nm (BMG Labtech, Durham, NC). Cell viability was calculated as a percentage of absorbance of the samples relative to untreated control.

Apoptosis assay: flow cytometry

Cells were stained using an annexin-V-FITC Apoptosis Detection Kit (Invitrogen) according to the manufacturer's instructions. Briefly, equal numbers of melanocytes (Mf0639 P4) were plated and treated with 500 or 900 μM of 4-TBP or MBEH for 1 hour. Floating and adherent cells were combined and subsequently incubated for 15 minutes with annexin-V-FITC and propidium iodide. Cells were acquired with a FACScanto (Becton Dickinson, SanJose, CA) and analyzed with Flowjo software (TreeStar, Ashland, OR). Cells in the FITC-positive and propidium iodide-negative fraction were quantified as apoptotic cells.

Fluorogenic substrate assay

Caspase-3 activity was quantified using a fluorogenic substrate (Chaturvedi *et al.*, 2006). Briefly, equal amounts of cultured melanocytes (Mf0861, P4) were treated with 500 or 900 μM of 4-TBP or MBEH for 1 hour. Cells were then lysed in 50 μl of lysis buffer (25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylamino]-1 propane sulfonate, and 2 mM EDTA) and protein concentrations were determined by Bradford assays. Equal amount of protein was mixed with assay buffer (25 mM HEPES, 5 mM dithiothreitol, and 100 μM of substrate Ac(N-acetyl)-DVED-AFC (7-amino-4-trifluromethylcoumarin) and the reaction was incubated at 37 °C for 2.5 hours. Fluorescence was determined as a measure of caspase-3 activity in a cytofluor multi-well plate reader with 360 nm as

excitation and 530 nm as emission wavelengths (Invitrogen). The enzyme activity was reported as nmoles per milligram of protein per hour.

Western blots

To investigate the expression level of pro-apoptotic factors, melanocytes cells were treated with 500 or 900 μM of 4-TBP or MBEH for 1 hour. Vehicle-treated cells served as control. Cells were washed in phosphate-buffered saline and incubated in lysis buffer (50 nM Tris, 2 mM EDTA, 150 mM NaCl, and 1% Triton-X-100) in presence of a protease inhibitor cocktail (Roche, Indianapolis, IN). Cell extracts were spun, and protein content was estimated in the supernatant according to manufactures' instruction using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Cellular proteins were electrophoresed in a 10% polyacrylamide gel and were transferred to a PVDF membrane (Millipore, Billerica, MA). The blots were incubated with a rabbit polyclonal anti-caspase-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit polyclonal anti-PARP antibody (Santa Cruz Biotechnology), or a mouse monoclonal anti-HMGB1 antibody (Novus biologicals, Littleton, CO), as well as with monoclonal anti-GAPDH (glyceral-dehyde 3-phosphate dehydrogenase) (Chemicon, Temecula, CA). Biotinylated anti-mouse IgG (Dako, Carpinteria, CA) or anti-rabbit IgG (Santa Cruz Biotechnology) antibodies were added followed by peroxidase-labeled streptavidin (Dako). Peroxidase activity was detected by ECL chemiluminesence in a film imager (Fuji, Stamford, CT).

Electron microscopy

For ultrastructural studies, melanocytes were seeded in multi-well Lab-Tek chamber slides (Nunc, Naperville, IL). Adherent cells were processed for electron microscopy as previously described (Boissy *et al.*, 1991). Briefly, cells were fixed in wells with half-strength Karnovsky's fixative in 0.2 M sodium cacodylate buffer at pH 7.2 for 30 minutes at room temperature. Cells were then treated with 1% osmium tetroxide containing 1.5% potassium ferrocyanide for 30 minutes and stained *en bloc* with 0.5% uranyl acetate for 30 minutes, dehydrated, and embedded in Eponate 12. Three areas of each culture were cut out of the Epon cast, mounted on Epon pegs, and sectioned on an RMC MT 6000-XL ultra-microtome. Ultra thin sections were then stained with aqueous solutions of uranyl acetate (2%) and lead citrate (0.3%) for 15 minutes each, and viewed and digitally photographed using a JEOL JEM-1230 transmission electron microscope (JEOL, Peabody, MA). Tissue-processing supplies were purchased from Ted Pella (Tustin, CA).

TUNEL assay

Biopsies (6 mm) obtained from skin tissue were cultured and treated as described under the organotypic culture heading. Unfixed 8-μm cryosections were initially incubated with primary antibody M2-9E3 to MART-1 (Covance, Dedham, MA). Sections were then fixed in 1% paraformaldehyde and stained for DNA fragmentation using an ApopTag Fluorescein *In situ* Apoptosis Detection Kit (Chemicon) according to the manufacturer's instructions. In brief, sections were incubated with dioxygenin-labeled oligonucleotides in presence of TdT (terminal deoxynucleotidyl transferase) enzyme to extend the 3′OH breaks of the DNA. FITC-labeled nucleotides were added, followed by phycoerythrin-labeled streptavidin (Dako). The sections were analyzed by confocal analysis with a Zeiss LSM-510 microscope (Zeiss, Thornwood, NY) after counterstaining with 4′, 6-diamidino-2-phenylindole (Invitrogen). The microscope offers different lasers used to detect the individual fluorochromes included in this assay. 4′, 6-diamidino-2-phenylindole fluorescence was identified using a Diode laser exciting at 405 nm. Phycoerythrin was monitored using a HeNE 633 laser. Detection of FITC was performed using a multiline-argon laser exciting at 488 nm.

Intracellular staining

Melanocytes treated with 250 μM of 4-TBP or MBEH for 72 hours were lifted with 0.5 mM EDTA. For permeabilization, cells were fixed in 2% paraformaldehyde, and washed with 0.03% saponin. The cells were incubated for 1 hour at 4 °C with monoclonal antibody Ta99 to TRP-1 (Covance), goat polyclonal antibody D-18 to TRP-2 (Santa Cruz Biotechnology), monoclonal antibody T311 to tyrosinase (Neomarkers, Fremont, MA) prepared in 0.3% saponin. Biotinylated anti-mouse IgG (Dako) or anti-goat IgG (Santa Cruz Biotechnology) were used as secondary antisera followed by phycoerythrin-labeled streptavidin (Dako). Flourescence data were acquired with a BD FACScanto flow cytometer (Becton Dickinson) and analyzed with Flowjo software (TreeStar).

Immunohistochemistry

Treated organotypic skin cultures were obtained as described above. Cryosections of 8-μm thickness were stained with monoclonal antibody Ta99 to TRP-1, goat polyclonal antibody D-18 to TRP-2, monoclonal antibody T311 to tyrosinase, monoclonal antibody M29-E3 to MART-1, monoclonal antibody NKI-Beteb to gp-100, or monoclonal 124 to Bcl-2 (Dako). Biotinylated anti-mouse (Dako) or anti-goat (Santa Cruz Biotechnology) were used in the second step followed with peroxidase-labeled streptavidin (Dako). Images were captured by an Olympus BX41 bright field microscope (Olympus, Center Valley, PA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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