Chemoprevention of Familial Melanoma

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LOYOLA UNIVERSITY CHICAGO

CHEMOPREVENTION OF FAMILIAL MELANOMA

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN MOLECULAR AND CELLULAR BIOCHEMISTRY

BY

VIDHYA HARIHARAN

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<tr>
<td>4-TBP</td>
<td>4-tertiary butyl phenol</td>
</tr>
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<td>MBEH</td>
<td>Mono benzyl ether of hydroquinone</td>
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<td>TRP-1</td>
<td>Tyrosinase related protein-1</td>
</tr>
<tr>
<td>TRP-2</td>
<td>Tyrosinase related protein-2</td>
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<tr>
<td>MART-1</td>
<td>Melanocyte antigen recognized by T cell-1</td>
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<tr>
<td>gp-100</td>
<td>Glyco protein 100</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>CDKN2A</td>
<td>Cyclin dependent kinase 2A</td>
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<tr>
<td>Ink4a</td>
<td>Inhibitor of kinase 4a</td>
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<td>ARF</td>
<td>Alternate reading frame</td>
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<td>CDK4</td>
<td>Cyclin dependent kinase 4</td>
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<tr>
<td>CDK6</td>
<td>Cyclin dependent kinase 6</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
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<td>CTLA4</td>
<td>Cytotoxic T-lymphocyte-associated protein 4</td>
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<tr>
<td>BCL-2</td>
<td>B-cell lymphoma 2</td>
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<td>MCL</td>
<td>Magnetic cationic liposomes</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PI3 kinase</td>
<td>Phosphatidyl inositol-3 kinase</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FITC</td>
<td>Flourescein isothiocyanate</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>KD</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
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<td>RT-PCR</td>
<td>Realtime polymerase reaction</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>Th mediated</td>
<td>T-helper cell mediated</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
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<tr>
<td>MAP kinase</td>
<td>Mitogen activated protein kinase</td>
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CHAPTER I

AN INTRODUCTION TO MELANOMA AND MELANOMA THERAPY

*Melanoma predisposition*

Melanoma, the malignant tumor of melanocytes, is the most aggressive malignancy of the skin. The occurrence of melanoma in the light-skinned Caucasian population has more than tripled in the last 20 years, and currently melanoma stands out to be the sixth most common cancer in the United States. In the year 2009, around 8650 deaths (5550 men and 3100 women) were reported as a result of melanoma in the United States (2). A history of skin cancer, presence of multiple benign or atypical nevi, immunosuppressive treatments, and exposure to ultra violet radiation all predispose to melanoma development, however, family inheritance still remains the most significant risk factor for melanoma (4). A meta-analysis conducted to identify inheritance patterns suggested that the presence of one first degree relative with melanoma increases the risk by 2.44 fold (5). A search for genetic abnormalities within families affected with melanoma identified primarily 2 genes (CDKN2A and CDK4) whose mutations are associated with increased melanoma susceptibility.

The CDKN2A gene located at chromosomal locus 9p21 was found to be mutated, hyper methylated or deleted in more than 75% of melanoma cell lines (6). The CDKN2a genetic locus codes for two distinct cell cycle regulatory proteins that are translated in
alternative reading frames (7). The $\alpha$ transcript encompassing exons 1$\alpha$, 2 and 3 codes for $p16^{\text{INK4a}}$ cyclin dependent kinase inhibitor while the $\beta$ transcript (exon 1$\beta$, 2, 3) encodes the protein $P14^{\text{ARF}}$ (p19 in mouse) (8-9).

$P16^{\text{INK4a}}$ interacts with CDK4 and CDK6 to inhibit the phosphorylation of retinoblastoma (RB) protein (10). The hypophosphorylated form of RB thus acts as a tumor suppressor and prevents progression of the cell cycle from G1 to S phase (11-12). Germline intragenic mutations in the $P16^{\text{INK4a}}$ gene were frequently identified in familial melanoma kindreds and melanoma prone families with an intact ARF function (13-15). Hence, INK4a is considered the key gene involved in predisposition to familial melanoma.

$P14^{\text{ARF}}$ prevents degradation of p53 by interacting with HDM2 (16) (17-18). The interaction of $p14^{\text{ARF}}$ with HDM2 inhibits the ubiquitin ligase activity of HDM2 on p53 thus preventing its export from nucleus (19). Since ARF is a splice variant of the INK4a transcript (16), germline mutations of ARF were expected in melanoma patients. Although insertions and deletions in the ARF exon 1$\beta$ with an intact INK4a were reported in a melanoma cell line, germline mutations were not initially identified among melanoma patients (20). The breakthrough came from two melanoma patients with germline ARF specific mutations. One patient displayed a 14kB deletion in exon 1$\beta$ (21), while the second patient had a 16kB insertion in exon 1$\beta$ (22) with intact $p16^{\text{INK4a}}$ in either case. Since then melanoma patients were screened for germline ARF mutations. Later a cluster of 5 different germline mutations was identified in the splice donor site of
exon 1β with intact INK4a and CDK4 exons (23). These splice variants were expected to impair ARF function; however, their functional role was not studied (24).

Germline mutations of cyclin dependent kinase 4 (CDK4) have also been identified in melanoma prone kindreds, however, the frequency of CDK4 mutation is rare (25-27). The CDK4 gene is present on chromosome 12q13, a region that is not rearranged or deleted in melanomas (28). Analysis of 31 families with heredity melanoma showed a C→T transition at nucleotide 297 in all 31 families with intact p16\textsuperscript{INK4a}. This C→T transition causes replacement of arginine with a cysteine at position 24 causing the kinase to be inactive (26, 29). Thus germline mutations or deletions in the CDKN2A or CDK4 loci predispose to melanoma.

Germline mutations in the “melanoma hotspot” genes act as first hit and loss of heterozygosity is the second and essential step to develop melanoma (30). Environmental stress in the form of excessive sun exposure is a significant contributing factor in generating a second level of genetic alterations for carriers of germline mutations since incidence of melanoma is markedly higher in people living at lower latitudes (31).

It is extremely intriguing how a mutation, for example in the CDKN2 locus, would primarily increase the odds of melanoma occurrence and not other tumors. To understand this, the senescence of mouse fibroblasts and melanocytes was studied upon single and/or double copy loss of the Ink4a/Arf gene. While no effect was identified in the senescence of fibroblasts upon Ink4a loss, the senescence of melanocytes was significantly affected with a single copy loss of Ink4a/Arf gene (32) (33). This suggests that Ink4a/Arf genes are primarily involved in cell cycle regulation. A similar effect was
observed in human melanocytes, where functional loss of $p16^{\text{INK4a}}$ and $p14^{\text{ARF}}$ had a significant effect on melanocyte senescence when compared with human fibroblasts (34). These results demonstrate the importance of INK4a/ARF genes in controlling regular cell cycle progression in melanocytes.
Fig. 1  The CDKN2A locus and cell cycle control.
Exons 1a, 2, and part of 3 combined to form the p16\textsuperscript{INK4a} transcript, while the exons 1b and 2 form the p14\textsuperscript{ARF} transcript. The p14 protein is shown to inhibit the function of MDM2 or HDM2, thereby preventing the degradation of p53. P16\textsuperscript{INK4a} interact with CDK4/6 to prevent the phosphorylation of RB. The hypo phosphorylated form of RB inhibits cell cycle progression (3)
Apart from germline mutations, various somatic mutations have been detected in melanoma patients. The most common somatic mutation present in more than 70% of melanoma patients are found within the BRAF gene (35). Germline mutations in BRAF have not been identified in melanoma patients; however, individuals with germline BRAF mutation develop cardio-facio-cutaneous syndrome, a rare genetic disorder affecting the face and head structure, but not melanomas (36-38). Among BRAF mutations, a phosphomimetic substitution in the kinase domain (valine to glutamic acid at amino acid 600) is the most frequently encountered (39). Such activating BRAF mutations were also observed in dysplastic nevi, suggesting that BRAF mutations play a role in early transformation of melanocytes. (40-43).

Other common mutations in melanoma are found within the phosphatase and tensin homolog (PTEN) and RAS genes. PTEN protein acts as a tumor suppressor by regulating the cell cycle through its phosphatase activity (44). PTEN dephosphorylates phosphatidyl inositol -3-phosphate (PIP3) resulting in the formation of PIP2 (44). This dephosphorylation is a crucial step that controls the activity of AKT, and loss of PTEN leads to accumulation of PIP3, thereby increasing AKT activity and cell survival (44). Allelic loss or altered expression of PTEN contributes to 20% and 40% of melanoma cases, respectively (45-46), although homozygous deletions are rarely observed.

The RAS (rat sarcoma) subfamily of proteins is small GTPases whose activation contributes to cell growth, differentiation and survival. Activating RAS mutations are common in other solid tumors, while only 10-15% of melanomas carry RAS mutations
The RAS subfamily consist of KRAS, NRAS and HRAS; among them NRAS mutations are found in melanomas while KRAS and HRAS mutations are rare (15).

As stated previously, to develop melanoma, more than one genetic alteration is required. These genetic alterations are either inherited or acquired. In many cases patients do present multi level genetic abnormalities such as mutations in the both p16\textsuperscript{INK4a} and BRAF (48). Thus it is evident that the genomics of melanoma is complicated, rendering the therapy of melanoma quite challenging.

**Melanoma therapy and resistance**

A key factor in the successful management of melanoma is early detection. Early malignant melanomas exhibit characteristic features including asymmetry, border irregularity, color variegation, or a diameter greater than 6 mm. These features are referred as the ABCD system of diagnosis (49). However, not all early melanomas exhibit any or all of these characteristic features. Around 5% of all melanomas are not pigmented and are misdiagnosed as squamous or basal cell carcinoma or inflammatory lesions (50-51). Early diagnosed melanomas have an excellent prognosis, however the survival rate drops rapidly once melanomas reach 3mm in thickness (49).

1. **FDA approved therapeutic approaches for melanoma**

Few FDA-approved treatments are available for metastatic melanoma. Dacarbazine (DITC) remains the only chemotherapeutic compound commonly used for metastatic melanoma. DITC targets the cancer cells by alkylating the DNA (52). Other therapeutics with FDA approval are Interferon-\(\alpha\)\(_{2b}\) (IFN- \(\alpha\)\(_{2b}\)) and Interleukin-2. IFN-\(\alpha\)\(_{2b}\) is used for stage III melanoma, showing a 10-20% improvement in patients’ relapse
free survival. However, it does not show any effect on melanoma related mortality (53). High dose IL-2 is used for patients in stage IV melanoma. Although short lived, IL-2 treatments are associated with severe toxicities but with 8-10% cure rate for melanoma (54-55).

2. Therapeutic approaches to target oncogenic pathways

Identification of signaling pathways in melanoma progression have opened up new areas of research to develop novel therapeutic agents. Discussed below are some of the key targets that are being explored alone or in combination with chemotherapeutic agents.

a. The MAP kinase pathway

In several cancers, the RAS-RAF-MAPK-ERK growth-signaling pathway has gained attention. A RAS farnesyl transferase inhibitor such as Tipifarnib was among the first of a set of agents that blocks the post-translational modification of RAS proteins, hence preventing their membrane localization. This drug was taken to phase II clinical trials in metastatic melanoma patients; however as the response rate was low, further clinical validation was prohibited (56).

Around 80% of melanoma patients do possess activating BRAF mutations (35, 57). Sorafenib was the initial BRAF inhibitor developed that targeted BRAF, CRAF, VGEH and PDGF receptor tyrosine kinases (58). Sorafenib showed relatively low potency towards melanoma when used as a single agent; however, it had a better effect when combined with other chemotherapeutic agents such as temozolomide or paclitaxel (59). The need for a more selective BRAF inhibitor led to the development of PLX4032.
This drug suppressed the activity of BRAF\textsuperscript{V600E} with approximately 30-fold increased selectivity over the wild type BRAF kinase (60). The drug is currently in phase III trials and has demonstrated remarkable clinical results (61-62). Unfortunately, melanoma patients develop resistance after 8-12 months of administering PLX4032. Recent studies performed to understand the drug resistance observed in melanoma patients has shown that PLX4032 blocks the growth of tumors that have BRAF mutations by selectively shutting down the activity of mutant BRAF monomers. Paradoxically, the activity of normal BRAF kinase in the cancerous and non-cancerous cells was shown to be activated by this drug. It was shown that this drug transiently elevates the expression levels of MEK and RAF in cells with wild type RAF, thus facilitating their survival (61, 63-64).

b. MEK kinases and PI3kinase

MEK kinase is downstream of the BRAF signaling pathway. Several MEK inhibitors have been tested in clinical trials in patients with advanced melanoma. PD0325901 is currently in phase I trials and the drug specifically inhibits the MEK1/2 mediated signal (65).

It is known that TOR acts as a major effector of the AKT oncogenic signaling pathway, which is the downstream of PI3 kinase (66-67). Small molecule inhibitor derivatives of rampamycin, targeting mTOR, demonstrated growth inhibitory effects in melanoma cell lines. However, similar effects were not observed in the clinical trials (68-70).
c. Cyclin dependent kinases

Mutation or deletion of CDKN2A along with increased activation of CDK2, CDK4 and CDK6, leads to hyperphosphorylation of RB and increased proliferation of melanoma tumor cells (15, 71). These findings prompted the development of several CDK inhibitors. Flavopridol is a well-characterized CDK inhibitor targeting the ATP binding pocket of CDK2, thereby blocking the G1 exit phase of the cell cycle. It was shown that this drug also blocks other cell cycle kinases such as CDK1, CDK2, CDK4 and CDK7 (72). This drug induced apoptosis in several cell lines but failed to be effective when taken to clinical trials (73). Since the cell cycle inhibitors primarily have cytostatic effects, combining such drugs with chemotherapeutic agents may increase their treatment efficacy. Other CDK inhibitors at various stages of clinical trials are UCN-01, CYC202, and BMS387032.

3. Other therapeutic approaches

a. Activation of apoptosis in melanoma cells

It is well known that resistance to apoptosis promotes tumor progression (74). Normal melanocytes express increased levels of several intrinsic and extrinsic anti-apoptotic proteins such as Bcl-xL and X-linked compared to other skin cells as a protective mechanism against UV-induced insult (75). Consequently, melanoma cells demonstrated increased resistance to apoptotic death compared to other tumor cells (76-77). Hence, attempts were made to induce apoptotic death in melanoma cells by blocking anti-apoptotic proteins. Oblimersen is an anti-sense agent targeted to mitochondrial Bcl-2. A phase I/II clinical trial suggested that this drug can sensitize
melanoma cells when combined with chemotherapeutic agent decarbazine (78). However, it did not improve the overall survival of melanoma patients in a randomized phase III clinical trial (79). The key limitation of this drug is that it can target cells other than melanoma cells.

b. Blocking of CTLA-4 using monoclonal antibody

Effective T cell activation requires co-stimulatory interaction between B7.1 or B7.2 on APC and CD28 on T cells (80). Upon activation, T cells increase the expression levels of CTLA-4. CTLA-4 would then compete for binding with B7 resulting in reduced TCR stimulation, IL-2 production or T cell proliferation (81-82). Monoclonal antibodies to CTLA block the interaction between B7 and CTLA4, thereby activating the T cells. As a result, peripheral tolerance to self-tissue is lost and anti-tumor responses are activated (83).

Anti-CTLA4 monoclonal antibody ipilimumab is in stage III clinical trials for metastatic melanoma (82, 84-85) and FDA approval was announced earlier this week. The main concern with using anti-CTLA-4 antibody is that it can induce septic shock, since the treatment overrides immune tolerance (86).

In spite of extended research in the field of melanoma therapy, progress has been limited as the tumor develops resistance to available therapies. This is particularly devastating for people with a hereditary predisposition to melanoma, since the onset of melanoma for these patients can occur at an early age (87). Therefore developing a preventive approach is critical. To design preventive therapies, understanding the functional role of precursor melanocytes is key.
Melanocytes and Melanogenesis

Melanocytes are neural crest-derived cells found in the basal layer of the epidermis (88). Apart from melanocytes, neural crest cells give rise to neurons, glial cells, adrenal medulla cells and cardiac cells (89). Precursors of melanocytes migrate from the neural crest to the dermis and finally settle down in the basal layer of the skin and the hair follicles. Resulting pigmentation patterns differ from species to species (88).

The key function of melanocytes is to protect the skin from harmful UV rays by producing a complex unstructured pigment called melanin (89). The process of melanin synthesis is restricted to melanocytes and takes place within lysosome-like organelles, the melanosomes (89). Depending on skin type, the synthesis of either of two types of melanin, red/yellow pheomelanin or brown/black eumelanin predominates. The amino acid tyrosine is the common natural substrate for eumelanogenesis and pheomelanogenesis (90-91). The process of eumelanogenesis as postulated by Raper and Mason (92-93) involves the conversion of tyrosine to DOPA (3,4-dihydroxyphenylalanine) and successively to dopaquinone, dopachrome, dihydroxyindoles, indolequinones and finally to eumelanin (90-91). Pheomelanin is a reddish-yellow polymeric pigment that is formed from 5-S-cysteynyldopa. The synthesis of pheomelanin over eumelanin is favored when the intramelanosomal cysteine concentration is $>10^{-7}$ M (90, 94). It is interesting to observe that eumelanin and pheomelanin significantly differ in their chemical structure and physical properties (95-96). Upon UV radiation, pheomelanin was observed to generate higher quantities of free radicals which can eventually lead to cellular lysis (97). This explains why people
producing increased pheomelanin in their skin have more severe sunburns, thereby increasing the odds of developing skin cancer due to DNA damage in melanocytes and keratinocytes.

The process of melanin synthesis involves several enzymes and proteins. Tyrosinase is the key, rate limiting enzyme of melanogenesis (98). It is a copper-containing oxido reductase that is involved in the oxidation of mono and dihydric phenols to their respective orthoquinones (98). The key feature of the tyrosinase active site is that it has two binuclear copper binding sites that are in close proximity to histidine residues which assist in the binding of molecular oxygen (1, 99). Depending on the valency of the copper ions, the active site of tyrosinase exists in oxy or met conformation. In the oxy conformation, the monovalent copper ions interact with an oxygen molecule yielding a peroxide. The other conformation, the met-tyrosinase, is the native form of tyrosinase containing bivalent copper ions and cannot bind with molecular oxygen. Two electron reduction of met-tyrosinase renders deoxy-tyrosinase in which the copper atoms are in the Cu$^{1+}$ oxidation state. Deoxy-tyrosinase readily binds dioxygen and returns the enzyme to oxy-tyrosinase conformation, which can oxidize phenolic substrates, and the cycle between de-oxy and oxy conformation continues in presence of oxygen. In case of a catecholic substrate, oxidation by oxy-tyrosinase returns the enzyme to met conformation. However, the enzyme returns back to oxy-conformation only in presence of a second catecholic molecule (1, 94). This could explain why phenolic substrates generate increased levels of toxic intermediates, while the toxicity is limited to some extent when catecholic substrates are used.
Fig. 2 Schematic diagram showing oxidation of catechol and phenol.
The diagram illustrates that phenol can be oxidized by tyrosinase in the oxy tyrosinase form, while catechol can be oxidized both by oxy and met tyrosinase form. Further, the conversion of deoxy and oxy tyrosinase form depends on the presence of oxygen and is reversible (1). Reproduced with permission.
Other enzymes and proteins involved in the process of melanogenesis are tyrosinase related-protein 1 (TRP-1), tyrosinase related protein-2 (TRP-2), melanoma antigen recognized by T cells (MART-1), and gp100. Tyrosinase related proteins are known to have similar structural features to tyrosinase such as having a transmembrane region, and two metal-binding regions, and a cysteine-rich epidermal growth factor motif, however they have a distinct catalytic role in the biosynthesis of the melanin (100). TRP-1 aids in proper folding of tyrosinase via chaperone-type interactions (101) and stabilizes tyrosinase by interacting with the cysteine-rich epidermal growth factor motif (100). A mutation in human TRP-1 will affect the activity of tyrosinase as well prevent its export from the endoplasmic reticulum (100-101).

Tyrosinase-related protein 2 (TRP-2) regulates melanin biosynthesis and metabolizes the toxic intermediates of the melanin synthetic pathway. The production of eumelanin versus pheomelanin depends on the enzymatic activity of TRP-2 (102). Active and accelerated levels of TRP-2 increases isomerization of DOPAchrome to DHICA thereby favoring the eumelanin production rather than the spontaneous conversion of DOPAchrome to DHI, which is a toxic intermediate thought to suppress the growth of cells (100, 102). Production of pheomelanin dominates when TRP-2 levels are lower because of diminished flow of dopaquinone through dopachrome to eumelanins (102).

Pmel17/gp100 is the scaffold protein that is delivered to the early melanosomes and undergoes proteolytic cleavage to form the fibrilar structure of melanosomes onto which melanin is deposited (103-104). MART-1 forms a complex with Pmel17 and its expression level affects the stability, structure and maturation of melanosomes (103).
The skin color depends upon the amount of melanin present. It is interesting to observe how people with lighter skin opt to darken their skin tone using the tanning salon, and people with a darker skin tone are using depigmenting agents to lighten their skin. Depigmenting agents are widely used in Asia. Also, it is recommended by dermatologists for hyperpigmentary skin conditions such as melasma (105).

Depigmenting agents

Depigmenting agents are categorized into two types. The first class of agents reduces the amount of melanin formation while the second class is cytotoxic to melanocytes. A number of tyrosinase inhibitors from natural and synthetic sources have been used extensively as skin lightening agents. Some of the commonly used skin lightening agents are:

1. Kojic Acid

Kojic acid is the most intensively studied metabolite of the fungus *Aspergillus oryzae*. Kojic acid blocks melanin synthesis by chelating the copper sites in tyrosinase enzyme, thereby inhibiting its phenolase activity. It is used extensively in the food industry to prevent the browning reaction of various food products and in the cosmetic industry to lighten the skin tone (106-108).

2. Flavanols

Flavanols are generally isolated from plants and most of them are found to be tyrosinase inhibitors. Flavanols competitively inhibit the oxidation of L-DOPA and act as a copper chelator in the active site of the tyrosinase enzyme (109-110). Some of the flavanols acting as tyrosinase inhibitors are nobiletin (5,6,7,8,3',4'-hexamethoxyflavone),
naringin (5,7,4'-trihydroxyflavanone), and neohesperidin (5,7,3'-trihydroxy-4'-methoxyflavone). However, the inhibitory strength of different flavanols were found to be less in comparison with with kojic acid (111-112).

Other skin lightening agents that are commonly used in the cosmetic industry include hydroquinone, soy, glabridin and glycolic acid. These agents are also tyrosinase inhibitors, and are involved in reducing the melanin content (113-114). The efficacy of depigmenting agents depends on how close they mimic substrates of the melanogenic pathway, in particular tyrosine. Competitive inhibition of tyrosinase is a common factor for skin lightening agents and melanotoxic agents; however, it has not been clarified how depigmenting agents mediate toxicity towards melanocytes. It has been hypothesized that melanotoxic depigmenting agents would be converted to reactive orthoquinones by tyrosinase, thus leading to destruction of melanocytes (115). Some of the agents that are reported to compete with tyrosine as well as mediate toxicity in melanocytes are 4-hydroxy anisole, curcumin, and 4-tertiary butyl phenol (116-117). Recently, the underlying mechanism of tyrosinase-catalyzed conversion of phenolic substrates was modeled using the FDA-approved depigmenting agent, mono-benzyl ether of hydroquinone (MBEH). MBEH was shown to interact with mushroom tyrosinase and generate reactive ortho-quinonones, thereby destructing the melanocytes (118). Further, the unstable ortho-quinonones were shown to undergo dimerization or coupling with thiol compounds such as glutathione, or with proteins such as bovine serum albumin. These mechanism might bear relevance to the pathogenicity of vitiligo where the tolerance to melanocyte antigens is lost (115). The hypothesis is that the oxidized quinones have a
greater tendency to bind with melanogenic enzymes. Binding of these small molecules to larger proteins alters their structure, thereby generating neoantigens and breaking immune tolerance. Since the process of melanogenesis is common between melanocytes and melanoma cells, it was proposed to target melanoma cells using cytotoxic depigmenting agents.

**Pro-Drug therapy using melanogenesis pathway**

Oxidation of phenols to orthoquinones is an essential step in the process of melanogenesis (98). Orthoquinones are highly reactive molecules and can covalently bind with proteins and nucleic acids (119). Limiting the diffusion of quinones (such as dopaquinone) with hydrophilic side chains through the melanosomal membrane, rapid cyclization of the orthoquinones to reduce the accumulation of toxic intermediates, and increasing the activity of melanosomal enzymes such as TRP-2 are some of the ways by which melanocytes regulate quinone toxicity (102, 119). Researchers explored above-mentioned regulatory mechanisms to design pro-drugs for targeting melanoma cells. Pro-drugs are inactive precursors that are metabolized or activated in the body to release or generate the active drug, preferably at the target site (1). Ideally, pro-drugs for melanoma therapy should evade hepatic metabolism, reach the tumor tissue, enter melanosomes to be oxidized to toxic quinones, re-enter the cytoplasm of melanoma cells and activate cellular lysis.

Two concepts were used to design pro-drugs targeting melanogenesis. They are referred to as the ‘*Achilles heel approach*’ and the ‘*Trojan horse approach*’. The ‘*Achilles heel approach*’ involves the use of tyrosine analogs with bulky side chains to
maximize the formation of orthoquinone intermediates by preventing the cyclization reaction. Since melanoma cells have a porous melanosomal membrane (120), it was thought that the newly formed toxic intermediates would be released into the cytosol. Among various compounds that were tested, 4-hydroxy anisole was found to be oxidized by tyrosinase into 4-methoxycatechol. The oxidized product was found to be cytotoxic to melanocytes and melanoma cells, and pilot clinical studies were conducted in melanoma patients (121-125). Unfortunately, the clinical trials had to be terminated as the pro-drugs themselves are toxic to other cell types, thus causing liver and renal toxicity (126-127).

In search of new cytotoxic agents, Fujita et al found that pheomelanin precursor 5-cysteyndopa is more effective than dopa in inhibiting melanocyte growth and proliferation (128). Subsequently, several new compounds were synthesized and assayed for toxicity towards melanoma cells (129-130). Among tested compounds, 4S-cysteaminylphenol (4S-CAP) induced significant depigmentation (80%) in guinea pigs, showed specific toxicity to melanin containing cells and prolonged the life of B16 tumor bearing mice (131-133). Further studies with radio-labeled 4S-CAP showed selective incorporation of the chemicals in tumors generated after challenging the mice with B16 melanoma cells (134). In spite of these promising results, the limited solubility and low LD$_{50}$ have thus far prevented the use of 4S-CAP in vivo.

In the field of cancer hyperthermia, cancer cells displayed a 10-fold increased affinity for positively charged magnetic nanoparticles compared with uncharged nanoparticles. These positively charged magnetic particles were then heated using an alternating magnetic
field to kill the target cells. In order to attain specificity, a similar approach was recently applied where 4S-CAP loaded magnetic cationic liposomes (MCL) were used therapeutically. Intratumoral injection of 4S-CAP loaded MCL was performed in B16-challenged mice and melanoma nodules were heated using an alternating magnetic field, thereby regressing the tumor volume (135). Due to extreme toxicity of 4S-CAP, derivatives of 4S-CAP are currently being screened to identify a less toxic compound with similar specificity. Some potential candidates are N-AC-4CAP (N-acetyl-4-S-cysteaminylphenol) and N-propionyl-4-S-cysteaminylphenol (N-Pr-4-S-CAP). Similar to 4S-CAP, its derivatives were reported to be specifically toxic to cells with tyrosinase activity, and further studies are being conducted (136-138). A key problem for the *Achilles heel* strategy is that the rate constant for the reaction of *ortho*-quinones with thiols exceeds that of other reactions (139). The cytotoxicity induced by modified substrates is dependent on depletion of thiols, more specifically glutathione (GSH), which is present in the cells at concentrations in the millimolar range. Although the idea of designing pro-drugs to target the melanogenesis pathway is unique, progress is hampered by the toxicity of the prodrugs themselves, and targeting these compounds to metastatic melanoma tumor remains a challenge.

The ‘*Trojan horse*’ is a tale in the Trojan war, where Greek soldiers entered the city of Troy by hiding in a huge horse. In the melanoma pro-drug therapy context, the anti-cancer drugs are tagged onto (hidden by) dopaquinone, the natural substrate of tyrosinase. Once the substrate becomes oxidized by melanogenic enzymes, the hidden anti-cancer drugs are released into the melanoma cells and melanocytes. Since this
approach uses the natural substrate of melanin synthesis, the oxidized ortho quinone undergoes intramolecular cyclisation. Thus, the oxidized products would not induce toxicity while the newly released anti-cancer drugs would target the melanocytes and melanoma cells. Agents such as phenol mustard, bis-ethylamine mustard and daunorubicin (daunomycin) have been tagged onto melanogenic substrates (117). This approach provides an built-in drug delivery mechanism selective for melanoma cells. Since melanoma cells express different levels of melanogenic enzymes, targeting every melanoma cells by this approach may be difficult to achieve.

Thus, it is clear that harnessing the melanogenic pathway for melanoma treatment is difficult to achieve, since the depigmenting agents should be used systemically to target the metastatic tumors. However, it would be interesting to explore the prophylactic potential of these depigmenting agents.

**Modes of cell death**

Cell death can be categorized in to three major types: apoptosis, necrosis and autophagy. Apoptosis is a programmed cell death exhibiting unique characters such as membrane blebbing, cell shrinkage, chromatin compaction, DNA fragmentation (140). The process of apoptotic death can be initiated by an external or internal signal. Upon initiation, specific enzymes known as caspases would be activated (140). Caspases are cysteine proteases that are in an inactive form and are activated upon death signal. In humans three major class of caspases do exist. They are known as initiator (caspase -8, -9, and -10), executioner (caspase -3, -6, -7), and inflammatory (caspase-1,-4,-5, -11, and -12). The initiator caspases would form complex with adaptor proteins and would undergo
self-proteolysis that would activate the downstream executioner. Inflammatory caspases are primarily involved in cleaving cytokines involved with inflammatory responses (141).

Autophagy is the second mode of cell death where cellular organelles were degraded by autophagic vesicles. The two-membrane vesicles were formed in the endoplasmic reticulum, engulf the cellular contents, and fuse with lysosomes to degrade the cellular contents. PI3 kinase pathway have been involved in the process of autophagy, however the complete process is yet to be identified (140).

The third mode of cell death is necrosis where cells undergo a sudden trauma, infection or inflammation. A necrotic cell death exhibit cellular swelling, rupture of cell membrane and release of cellular organelles. Although necrosis is generally defined as un-programmed cell death, studies have identified that mitochondrial swelling, lysosome rupture with activation of calpain and cathepsin have been identified in necrotic death (140).

Need for prevention

Removal of precursor tissue or precursor cells that can otherwise undergo malignant transformation is a concept for preventive treatment. For example, women with germline mutations within the BRCA genes are at a high risk of developing breast or ovarian cancer (142). Such women sometimes undergo elective surgery as a preventive measure towards potential future cancer development (143). Such surgical intervention is not feasible for familial melanoma patients as the precursor cells (“melanocytes”) are dispersed throughout the skin. However, specific removal of melanocytes from the skin by methods similar to those described above can potentially serve as a prophylactic
treatment, similar to elective mastectomy for women predisposed to breast cancer. Inducing a vitiligo like condition would then serve as a potential preventive treatment for familial melanoma, since an intriguing dichotomy exists between vitiligo and melanoma.

**Vitiligo and melanoma-striking similarities**

Vitiligo is an autoimmune disease affecting 0.5% of the world population (144-145). The association of vitiligo with other autoimmune diseases like Hashimoto's thyroiditis suggested the involvement of an immune response in the pathogenesis of the disease. It is extremely interesting to find striking similarities between vitiligo and melanoma.

Vitiligo is most devastating to ethnicities associated with darker skin tones, where the contrast between unaffected and lesional skin is highly apparent. Melanoma on the other hand, preferentially frequents people of lighter skin tones. Whereas more males than females are struck by melanoma, vitiligo is more prevalent among females (unpublished observation). The etiology of either disease involves a hereditary component. In vitiligo this is supported by the fact that approximately 50% of patients are able to identify first degree relatives with the disease (146), whereas familial melanoma has been assigned to particular genes including the Ink4A/Arf genes encoded on chromosome 9 (147). Loss of melanocytes from the epidermis in vitiligo is associated with immune infiltrates in actively depigmenting skin, consisting of T cells with a decreased CD4/CD8 ratio, dendritic cells and macrophages (145). These T cells are, at least in part, responsive to melanosomal antigens also targeted by T cells infiltrating melanoma tumors and targeted by tumor vaccines in clinical trials (145, 148). Melanoma
patients responding to vaccine therapy may experience secondary depigmentation, which is considered a positive prognostic factor reflecting activated immune responses to the tumor (149). On the other hand, T cells isolated from vitiligo patient skin can express a higher affinity for their cognitive antigen than tumor infiltrating T cells. This renders vitiligo T cells an optimal source for the development of transgenic TCR based therapy for melanoma (150). The curious observation that the same melanosomal antigens are optimal target molecules for cytotoxic T cells in either disease drives the concept that vitiligo may offer protection from subsequent development of melanoma. This is the fundamental ground for inducing vitiligo in melanoma patients as proposed in this project. As the development of melanoma can predispose patients to vitiligo, epidemiologic data will not necessarily reveal such protective effect (151).

**Novel chemopreventive therapy using topical bleaching phenols**

The melanogenic pathway offers a unique means of generating toxic compounds specifically in cells of the melanocyte lineage with great potential towards the treatment of melanoma. As explained above, systemic use of melanotoxic agents for melanoma therapy led to renal and liver toxicity (121). Therefore, these phenolic agents are better used in melanoma therapy by topical application. However, melanoma tumors themselves were fully resistant to these treatments. Generally, tumor cells were known to have increased levels of ATP-dependent ABC transporters that offer protection against various therapies by pumping out the toxic drugs that affect their growth (152). This suggests that melanoma cells can pump the prodrugs or subsequently generated toxic quinones out of the cell, thereby developing resistance. Similar observations were made by van den
Boorn et al, where topical treatment of MBEH alone on C57BL/6 mice in a therapeutic setting did not have any effect on already established B16 melanomas (153). Hence, using topical phenolic agents to target melanocytes rather than melanoma cells will better serve as a preventive treatment for people with a predisposition to melanoma, and those at risk for recurrent disease.

Phenolic agents 4-tertiary butyl phenol (4-TBP) and mono benzyl ether of hydroquinone (MBEH) were initially identified as causative agents in vitiligo-like pathogenesis (154-155). Although 4-TBP and MBEH were reported to induce depigmentation, it was not known whether these agents are toxic to melanocytes. *In vitro* studies revealed that 4-TBP is specifically cytotoxic to melanocytes in comparison with keratinocytes, while fibroblasts displayed a similar level of sensitivity (156). Additionally, 4-TBP was shown to induce apoptotic destruction of melanocytes (116). Since 4-TBP mimics the structure of tyrosine, it could subsequently be demonstrated that 4-TBP competitively inhibits tyrosinase activity at concentrations that do not impact on melanocyte viability (116).

Currently, MBEH is the only FDA approved drug within US to remove remaining pigmentation in advanced vitiligo patients in order to maintain a uniform skin tone (157). However, its working mechanism is not known.

**The aim of this project is to identify whether topical depigmenting agents can be used in the prophylactic treatment for people with a predisposition to melanoma.** Such protection can be offered in a two-tiered approach by eliminating precursor cells of the melanocyte lineage that can otherwise malignantly transform and by eliciting an
immune response to distant melanocytes not directly exposed to bleaching agents, as well as to melanoma cells that share expression of melanocyte differentiation antigens. These questions will be addressed in the following chapters.

Chapter 2 describes whether MBEH is cytotoxic specifically to melanocytes. Following this, the mode of cell death mediated by MBEH is identified in comparison with bleaching agent 4-TBP. Further, the potential involvement of melanogenic enzymes in depigmentation is addressed by correlating sensitivity to MBEH with basal expression levels of melanogenic enzymes and melanin content among melanocyte monocultures. Finally, this study serves to understand whether treatment with bleaching agents would generate reactive oxygen species in an in vitro setting.

In Chapter 3, the immune aspects mediated by 4-TBP and MBEH are studied in human explant cultures followed by treatment of wild type and k14-SCF mice. The immune effects of bleaching agents 4-TBP and MBEH were studied initially in normal human skin, in order to quantify Langerhans cell emigration in response to either agent. Following this, the infiltration of immune cells was studied in the mouse skin upon bleaching agent induced depigmentation. Finally, the nature of the immune response mediated by MBEH was identified by assessment of cytokine profiles in the treated skin.

In Chapter 4 attempts to generate a spontaneous pigmented melanoma mouse model are described. This was attempted by crossing Tyr HRAS- Ink4a/Arf/- albino mice (generating non-pigmented tumors) and k14-SCF epidermally pigmented mice. The features of resulting offspring were tested by genotyping, following tumor development and assessing melanoma marker expression in resulting tumors. Finally, the immune
component of anti-tumor effects mediated by MBEH was tested by pre-treating k14-SCF mice and challenging them with B16 melanoma tumors.

Chapter 5 contains the discussion section of all work performed and provides the overall conclusions of this study, as well as describing potential follow up studies to newly arising research questions.

In conclusion, this study explores novel prophylactic treatment opportunities using topical bleaching agents 4-TBP and MBEH of potential use for people with a predisposition to melanoma.
CHAPTER II
WORKING MECHANISM OF MBEH

Abstract

Monobenzyl ether of hydroquinone (MBEH) is an FDA-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, demonstrating that the death pathway is non-apoptotic. Release of HMGB1 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, whereas 4-TBP reduced the expression of the same. Further, generation of reactive oxygen species was observed only upon MBEH treatment. 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.

Introduction

Vitiligo, a chronic pigmentary disorder, results from a T-cell mediated autoimmune response towards melanocytes (158). Patients with advanced vitiligo can opt to biochemically remove remaining pigmentation (159). For depigmentation therapy, new methods are gradually becoming available including the use of 4-methoxy phenol combined with Q switched ruby laser or cryotherapy (160). However, monobenzyl ether of hydroquinone (MBEH) remains the only drug FDA approved for depigmentation therapy within the United States (157). The working mechanism of MBEH remains unexplored, although MBEH is believed to remove melanocytes from the skin as it generally mediates permanent depigmentation (157). However, in some patients repigmentation has been observed after treatment (161).

The depigmentation process that follows exposure to phenolic agent 4-tertiary butyl phenol (4-TBP) is better understood. 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 (162), the cytotoxic effects were found to be independent of tyrosinase activity (156, 162). 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 (163). Normal melanocytes were shown to down-regulate the levels of microphthalmia-
associated transcription factor and TRP-1 in response to 4-TBP as a protective mechanism (163). Finally, 4-TBP is shown to activate apoptosis in melanocytes (156).

Phenolic agents like 4-hydroxyanisole, 4-TBP, and MBEH are thought to be oxidized and converted to reactive orthoquinones upon interacting with tyrosinase (115). Recent studies by Manini et al have shown that MBEH can bind with mushroom tyrosinase and generate ortho-quinones (118). Therefore, it can be expected that phenolic agents can generate reactive species in melanocytes.

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 (164). As an early signal, cells undergoing apoptosis display surface expression of phosphatidyl serine and are opsonized for phagocytosis by phosphatidyl serine receptor expressing macrophages (165-166). Alternatively, necrosis is an unregulated form of cell death characterized by cytoplasmic swelling and disintegration of the plasma membrane and cellular organelles (164). Necrotic cells stimulate an inflammatory response by releasing immune stimulatory agents like DNA binding High Mobility Group Box-1 protein (HMGB1) and heat shock proteins (167-168), and necrosis is therefore considered a strong inducer of immune responses (168).

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 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 upon treatment with 4-TBP or MBEH were identified by FACS analysis of cultured cells. Finally, this study would also understand whether treatment with bleaching agents would generate reactive species in an in vitro setting. 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.

**Materials and methods**

**Cell culture**

Cultures of normal human melanocytes, keratinocytes and fibroblasts were established from otherwise discarded foreskin tissue obtained after routine circumcision from the University of Chicago and Loyola medical center. All studies performed with
human tissue were carried out in adherence with the Declaration of Helsinki and were approved by the Institutional Review Board of the institution where circumcisions were performed. Dermo-epidermal separation was performed by incubation with 0.5 mg/ml thermolysin (Sigma, ST Louis, Mo). Epithelial cells were further separated by incubation in 0.1% trypsin. Cells or dermal tissues 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 2mM Glutamine (Invitrogen, Carlsbad, CA), 100 IU/ml penicillin, 100 µg/ml streptomycin 100 µg/ml amphotericin (Invitrogen), 0.1 mM IBMX (Sigma), 10 ng/ml TPA (Sigma), and 1% Ultroser G (Pall Biosepra- Cergy, France).

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

Normal human keratinocytes were cultured in growth medium with M154 basal medium (Cascade Biologicals, OR), supplemented with human keratinocyte growth supplements (Cascade Biologicals) with 100 IU/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml amphotericin (Invitrogen) and immortalized with HPV16 E6 and E7 retroviral constructs as described previously to allow prolonged passaging (169).

**Organotypic culture**

Organotypic culture was performed as described previously (170). In brief, 6mm biopsies were punched from otherwise discarded foreskin tissue obtained after routine
circumcision. The explants were cultured in Dulbecco's Minimal Essential Medium containing 10% inactivated normal human serum at the air-liquid interphase in 0.4 micron transwell plates with epidermal side facing up (Corning, Teterboro, NJ). After treatment with 5µl of 250mM of 4-TBP or MBEH for 24 hours, explants were embedded in 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 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/well were plated in triplicate wells of a 96 well plate to attach overnight. Cells were treated either with vehicle alone or with 250 µM, 500µM or 900 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% DMSO and mixed with 70% ethanol for a stock concentration of 250 mM. The use of a vehicle control refers to the use of 20% DMSO in 70% ethanol. MTT reagent ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) was added to the cells and incubated in a 37°C humidified chamber for 4 hours. Tetrazole is converted to formazan in 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 Inc, Durham, NC).
Cell viability was calculated as a percentage of absorbance of the samples relative to untreated controls.

**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 500µM or 900µM of 4-TBP or MBEH for one hour. Floating and adherent cells were combined and subsequently incubated for 15 minutes with annexin-V-fluorescein isothiocyanate and propidium iodide (PI). Cells were acquired with a FACScanto (Becton Dickinson, SanJose, CA, USA) and analyzed with Flowjo software (TreeStar, Ashland, OR). Cells in the FITC-positive and PI-negative fraction were quantified as apoptotic cells.

**Fluorogenic substrate assay**

Caspase-3 activity was quantified using a fluorogenic substrate. Briefly, equal amounts of cultured melanocytes (Mf0861, P4) were treated with 500µM or 900µM of 4-TBP or MBEH for 1 hour. Cells were then lysed in 50µl of lysis buffer (25mM HEPES, 10% sucrose, 0.1% CHAPS, and 2mM EDTA) and protein concentrations were determined by Bradford assays. An equal amount of protein was mixed with assay buffer (25mM HEPES, 5mM DTT, and 100 µM of substrate Ac(N-acetyl)-DVED-AFC (7-amino-4-trifluromethylcoumarin) and the reaction was incubated at 37ºC for 2.5 hours. Fluorescence was determined as a measure of caspase-3 activity in a cytofluor multi-well plate reader with 360nm as excitation and 530nm as emission wavelengths (Invitrogen). The enzyme activity was reported as nmoles/mg protein/hour.
Western Blots

To investigate the expression level of pro-apoptotic factors, melanocytes cells were treated with 500μM or 900uM of 4-TBP or MBEH for 1 hour. Vehicle treated cells served as control. Cells were washed in PBS and incubated in lysis buffer (50nM Tris, 2mM ethylene diamine tetracetic acid (EDTA), 150mM 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 manufacturer’s instructions 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 Inc, Santa Cruz, CA), a rabbit polyclonal anti-PARP antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA), or a mouse monoclonal anti-HMGB1 antibody (Novus biologicals, Littleton, CO) as well as with monoclonal anti-GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) (Chemicon, Temecula, CA). Biotinylated antimouse IgG (Dako) or anti rabbit IgG (Santa Cruz Biotech Inc, Santa Cruz, CA) antibodies were added followed by peroxidase labeled streptavidin (Dako, Carpinteria, CA). Peroxidase activity was detected by ECL chemiluminescence in a film imager (Fuji, Stamford, CT).

Electron Microscopy

For ultrastructural studies melanocytes were seeded in multi-well Lab-Tek chamber slides (Nune, Naperville, IL). Adherent cells were processed for electron microscopy as previously described (171). 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 min at room temperature. Cells were then treated with 1% osmium tetroxide containing 1.5% potassium ferrocyanide for 30 min and stained en bloc with 0.5% uranyl acetate for 30 min, 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 ultramicrotome. Ultra thin sections were then stained with aqueous solutions of uranyl acetate (2%) and lead citrate (0.3%) for 15 min each, and viewed and digitally photographed using a JEOL JEM-1230 transmission electron microscope. Tissue-processing supplies were purchased from Ted Pella (Tustin, CA, U.S.A.).

**TUNEL labeling**

Six millimeter biopsies 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- Temecula, CA) according to the manufacturer’s instructions. In brief, sections were incubated with dioxygenin-labeled oligonucleotides in presence of TdT enzyme to extend the 3’OH breaks of the DNA. Fluorescein isothiocyanate (FITC)-labeled nucleotides were added, followed by phycoerythrin (PE)-labeled streptavidin (Dako, Carpinteria, CA). The sections were analyzed by confocal analysis with a Zeiss LSM-510 microscope (Zeiss, Thornwood, NY) after counterstaining with 4′, 6-diamidino-2-phenylindole (DAPI) (Invitrogen). DAPI fluorescence was identified at 405 nm using a Diode laser. PE was
monitored using a HeNE 633 laser. Detection of FITC was performed using a multiline-argon laser excited at 488nm.

**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 4º C with monoclonal antibody Ta99 to TRP-1 (Covance, Dedham, MA), goat polyclonal antibody D-18 to TRP-2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), 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 Inc.) was used as secondary antisera followed by PE labeled streptavidin (Dako). Fluorescence data were acquired with a BD FACScanto flow cytometer (Becton Dickinson) and analyzed with Flowjo software (TreeStar).

**Measurement of reactive oxygen species**

2',7’Dichlorodihydrofluorescein diacetate (H₂DCFDA) (Molecular probes, CA), is a cell- permeable nonfluorescent compound. Upon oxidation by reactive oxygen species, H₂DCFDA emits green fluoresence (517-527nm) when excited by a blue light(.492-495nm) Melanocytes (Mf0639) were plated in 96 well plates at 10,000 cells/ well (Corning, Teterboro, NJ). Cells were treated with 25µM of H₂DCFDA reconstituted in melanocyte media and incubated for 45 minutes at 37ºC. After incubation, cells were washed with PBS, treated with 500 and 900µM of 4-TBP or MBEH, and followed over time for ROS generation. Emission of green fluorescence at 530nm was quantified and
the amount of ROS generation was calculated at different time points. Treatment with 100µM of H₂O₂ was used as a positive control.

**RESULTS**

*Cytotoxicity quantification*

The viability of cultured human primary melanocytes, fibroblasts and keratinocytes was determined through MTT assays after treating with 250 µM, 500 µM and 900 µM of 4-TBP or MBEH for 24 hours. In Fig.3, data are shown for a representative experiment of 2 performed. Vehicle treatment served as a control for all cell types. In Fig.3a, the viability of melanocytes was reduced by 26 % and 67 % in response with 250 µM 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 Fig.3b, 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 treatments at 250 or 500 µM concentrations. The viability of keratinocytes remained unchanged upon 250 µM exposure, while at 500 µM the viability was reduced only by 21 % and 31 % upon 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.
Fig. 3 Cytotoxicity of MBEH and 4-TBP towards 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/cell, fibroblasts (Ff0201 P4) and keratinocytes (Kf0180 P14) were treated with 250, 500 or 900 µM of 4-TBP (Fig.3a) or MBEH (Fig.3b) 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 where ‘*’ indicates p<0.05 and ‘**’ indicates p<0.01
for melanocyte viability in comparison with keratinocytes after 250 and 500 µM treatment of 4-TBP and MBEH treatment.
**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. PI-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 Fig. 4. Since melanocytes are more sensitive to MBEH than to 4-TBP as shown in Fig.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. Fig. 2 further confirms that no apoptosis was observed upon treatment with MBEH. Cells treated with vehicle alone served as controls for both treatments.
Fig. 4  AnnexinV staining of MBEH and 4-TBP treated cells

Melanocytes were treated with 500 or 900 µM of 4-TBP or MBEH (solid line) or vehicle alone (grey line) for 1 hour. FACS analysis of annexin-V staining, gating out PI stained (dead) cells, demonstrated that 4-TBP but not MBEH induced apoptotic cell death.
**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 upon cleavage. In a representative experiment shown in Fig. 5a, 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 Fig. 5b further confirmed that MBEH treatment did not induce caspase-3 cleavage, whereas 4-TBP treatment did induce cleavage of pro-caspase into active caspase. Cleavage of poly (ADP-ribose) polymerase (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 (Fig. 5c).
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 (nmoles/mg/hour) was quantified based on the amount of fluorescence emitted upon 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 following 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), while MBEH treatment did not induce PARP cleavage.
**Analysis of necrotic changes**

The morphological changes of cultured melanocytes in response to 4-TBP and MBEH were studied by light microscopy. Melanocytes were treated with 250 µM of 4-TBP and MBEH for 24 hours or with 900 µM for 1 hour, and structural alterations were observed. Representative figures are shown in Fig. 6a. Both 4-TBP and MBEH induced cell detachment. With 4-TBP apoptotic changes such as perinuclear vacuolization, dendrite retraction and membrane ruffling were observed as shown by representative arrows. MBEH treated cells however, demonstrated no apparent signs of apoptosis. Vehicle treated cells showed no significant morphological changes compared with untreated control cells. Following this, the ultrastructural changes in response to 4-TBP and MBEH exposure were examined by electron microscopy. Physiologic changes in treated cells were most readily demonstrable at the 1mM concentration for 1 hour, as shown in representative images of individual cells in Fig. 6b. 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 vacuolization, 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). Also, 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 upon MBEH treatment alone further confirming the necrotic pathway (Fig. 6c).
**Fig. 6 Analysis of necrotic changes**

a) Melanocytes Mf0632 P4 were treated with 250 µM of 4-TBP or MBEH for 24 hours, or 900 µM 1 hour and morphological changes were observed using a light microscopy. 4-TBP treated cells demonstrate changes such as membrane blebbing (long arrows), dendrite retraction (stars) and perinuclear vacuolarization (short arrows), MBEH treatment cells show non-adherent cells (arrowheads), and dendrite retraction (stars). (b) Morphological changes in melanocytes treated with 1mM 4-TBP or 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. Scale bars equal to 2 microns. (c) Western blot analysis of melanocyte (Mf0930 P2) supernatant to detect 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.
Confocal analysis of DNA fragmentation in exposed skin

DNA fragmentation in treated explants was analyzed by TUNEL staining. Human skin explants of 6mm in diameter were treated with 5µl of 250 mM of 4-TBP or MBEH for 1 day. Control explants were treated with vehicle alone. Cryosections were immunostained with APC-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 DAPI (4’, 6-diamidino-2-phenylindole) to identify cell nuclei. Stained sections were analyzed by confocal microscopy (Fig. 7). Similar to control samples, MBEH treated explants demonstrated 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.
Fig. 7 DNA fragmentation within MBEH or 4-TBP treated skin

Apoptosis was shown in confocal microscopic images of explant cultures exposed to 5µl 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. Scale bar is equal to 20 microns.
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 Fig. 8a, a positive correlation between cellular melanin content and viability following MBEH treatment was observed, with a correlation coefficient of 0.6. By contrast, melanin content did not correlate with cell death mediated by 4-TBP. In Fig. 8b, expression levels of melanogenic enzymes tyrosinase, TRP-1 and TRP-2 are 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 demonstrated 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.
Fig. 8 Role of pigmentation upon MBEH-mediated death

(a) Melanocytes were treated with 500 µM of 4-TBP or MBEH for 24 hours and cell viability was quantified with 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 upon MBEH exposure from left to right. The melanin content of each melanocyte culture was reported in pg/cell underneath the culture name.
**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 Fig. 9, 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.
Fig. 9 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 which was set as 100%.
Measurement of ROS generation

Melanocytes (Mf0639) were loaded with 25 μM of DCF, washed and then treated with 900μM of 4-TBP or MBEH and followed over time for ROS generation. Emission of green fluorescence at 530nm was quantified as amount of ROS generation over various time points. 100μM of H2O2 was used as a positive control. Fig. 10 shows a time-dependent increase in free radical generation after 60 minutes and 30 minutes with 500 and 900 μM treatment of MBEH, respectively. 4-TBP treatment did not induce any free radical generation. This indicates that free radical generation might be an upstream event of necrotic melanocyte death upon MBEH treatment. Experiment was repeated thrice in different melanocyte cultures and a representative experiment is shown in Fig.10.
Fig. 10 Quantification of ROS generation in 4-TBP or MBEH treated melanocytes

Green fluorescence emitted by DCF was quantified as a measure of ROS generation in melanocytes treated with 500 and 900 µM of 4-TBP or MBEH for 1 hour. MBEH induced time-dependent generation of free radicals both at 500 and 900µM, while no ROS was detected upon 4-TBP treatment.
Discussion

MBEH (10-20%) is used in dermatology clinics within the United States for depigmentation treatment of patients with advanced vitiligo (157). The current studies are the first to report on the underlying mechanism of depigmentation of human skin mediated by MBEH. Viability assays demonstrated that similar to 4-TBP, MBEH induces specific toxicity towards melanocytes as previously suggested by studies in mice (162, 172). At the same time, fibroblasts and melanocytes are equally sensitive to both agents, suggesting that neither agent should be used for systemic administration, thus limiting their use to topical application. Previous studies have reported that the mechanism of melanocyte destruction by 4-TBP is through apoptosis (156). 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. While 4-TBP treatment induced perinuclear vacuolarization and membrane ruffling, MBEH treatment induced disintegration of the cellular membrane and release of cellular contents as observed by light microscopy. 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 phosphatidylserine and DNA fragmentation. Electron micrographs confirmed the presence of necrotic changes including disruption of the plasma membrane and the nuclear membrane. Furthermore, release of HMGB1 was observed following MBEH
treatment alone, further emphasizing the same fact. Previous studies have demonstrated that active PARP is required for the release of HMGB1 (173). This explains the lack of HMGB1 release upon 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 (168, 174). 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 (175). 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 demonstrated that MBEH induces necrotic death in human melanocytes.

Correlative studies between melanin content and melanocyte sensitivity upon 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 (118). In this regard, MBEH appears to be converted into a quinone product upon interaction with tyrosinase (118). The accumulation of such quinone products may
induce toxicity selectively in melanocytes (176). Alternatively, the possibility should be considered that MBEH preferentially enters melanocytes as previously demonstrated for 4-hydroxy anisole in melanocyte-keratinocyte co-cultures (121). 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 staining upon MBEH treatment may in part explain the repigmentation observed in some patients following depigmentation therapy (157), 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 upon interaction with increasing levels of melanosomal enzymes. This observation contrasts with the observed decrease in TRP-1 levels associated with increased viability upon 4-TBP exposure (Manga and Boissy, 2006). Overall, chemical exposure was accompanied by opposing consequences for the expression of melanosomal enzymes both \textit{in vitro} and \textit{ex vitro}.

Oxidative stress is among possible causative factors in vitiligo (177). In this respect, vitiligo patients demonstrate modified levels of superoxide dismutase and catalase within the skin (178). Small nucleotide polymorphisms (SNPs) were identified
within the active site of catalase in some vitiligo patients. Modeling these SNPs demonstrated that they can affect catalase activity and render patients more sensitive to hydrogen peroxide (179). Also recently, the group of Schallreuter has identified upregulated activity of xanthine dehydrogenase/xanthine oxidase, and increased abundance of its metabolite allantoin in vitiligo epidermis (180). This can be ascribed to previously observed increases in H$_2$O$_2$ in vitiligo skin reported by the same group. H$_2$O$_2$ also impacts epidermal acetylcholine esterase activity and conversion of tryptophan to 5-HT (181). If serotonin synthesis is affected at a more systemic level, such findings can even impact on depression and suicidal tendencies observed in vitiligo patients. We have further reported significant increases in catechol-O-methyl transferase in vitiligo skin (182). Taken together, there is evidence of oxidative stress in vitiligo skin, and analogous to other diseases the presence of highly reactive oxygen radicals is likely to alter the antigenic uptake of melanocytes (Chiang et al, 2008). Such changes can drive an immune response to melanocytes. In this respect, it has been duly shown that minor modifications in differentiation antigens otherwise avoided as self-molecules can render such molecules immunogenic and drive autoimmune responses to unmodified variants of the same molecules. This principle was used to develop a mouse model of autoimmune vitiligo using human TRP-2 vaccines, for example. The vaccine elicits an immune response to the human protein that expands to include shared epitopes. ROS generation upon MBEH alone suggest that treatment with MBEH could be a potent activator of immune response
since oxidized quinones are expected to react with proteins and peptides at a higher affinity, thereby affecting their recognition by infiltrating T cells (183).

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. In part the explanation may be found in the different side chains to either phenolic agent and resulting differences in molecular structure potentially imposing differential reactivity towards melanocytes. Both 4-TBP and MBEH are specifically cytotoxic to melanocytes upon topical application with vastly different physiologic consequences that favor 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 (157). It may be possible to identify novel 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 immunologic responses to dying melanocytes, which can impact on the permanent features of depigmentation.
MBEH is specifically cytotoxic to melanocytes similar to 4-TBP. The mode of melanocyte death mediated by MBEH is necrosis, while 4-TBP induces apoptotic melanocyte death. In addition, MBEH generates ROS in melanocytes, not observed upon 4-TBP treatment. Finally, presence of melanin reduces the toxic effect mediated by MBEH and not 4-TBP. Thus the differential death mechanism mediated by bleaching agents 4-TBP and MBEH could mediate a differential immune response in melanocytes.
CHAPTER III

IMMUNE ASPECTS OF DEPIGMENTATION TREATMENT

Abstract

Phenolic agents 4-tertiary butyl phenol and monobenzyl ether of hydroquinone are preferentially cytotoxic to melanocytes and are known to induce vitiligo-like pathogenesis in an occupational setting. Since they are toxic to melanocytes, the therapeutic potential of these phenolic agents in melanoma treatment was explored by quantifying the sensitivity of melanoma cells to either agent by in vitro cytotoxicity assays. Initiation of an immune response mediated by 4-TBP or MBEH was studied in treated explant cultures where the migration of Langerhans cells into the dermal regions was quantified by immunohistochemical analysis. Since explant cultures cannot be used to study the influx of effector T cells from the lymph node further immunological studies were performed in an in vivo setting. To assess the depigmenting potential of bleaching agents in vivo, 4-TBP and MBEH were topically applied to C57BL/6 wild type as well as k14-SCF transgenic, epidermally-pigmented mice. Further, the influx of T cells, immigration of macrophages and cytokine expressions were measured in treated mouse skin. MTT assays showed melanoma cells lines 888, 624.38, A375 and M14 were relatively insensitive to either agent in comparison with melanocytes. Further, a lack of
expression of melanocyte markers TRP-1 and tyrosinase corresponded with increased melanoma cell line resistance to either agent, suggesting the involvement of melanosomal enzymes in sensitizing melanoma cells to treatments. Melanoma cells being resistant to 4-TBP and MBEH in vitro, the therapeutic effects of these drugs are likely limited, further supporting the need for a prophylactic approach to treatment for people with a predisposition for familial melanoma. Since MBEH treatment induces necrotic melanocytic death, an elevated level of immune response was expected in comparison with 4-TBP treatment. A significant increase in the migration of Langerhans cells towards the dermis only upon MBEH treatment suggested the selective elicitation of an immune response. MBEH also induced significant skin depigmentation in both strains, not observed upon 4-TBP treatment. Cytokine expression patterns in MBEH-treated skin support activation of a Th1-mediated immune response corresponding to an influx of T cells and macrophages. These data support that MBEH activate an immune response while no immune reaction was observed upon 4-TBP treatment.

Introduction

Monobenzyl ether of hydroquinone or MBEH is currently in use as an FDA approved agent to induce depigmentation in vitiligo patients (184). Depigmenting qualities of the compound were first described following attempts to identify a cause for skin depigmentation in an occupational setting. This led to the description of ‘occupational vitiligo’ as a separate disease category for patients undergoing gradual depigmentation in a work related setting (154, 185). Several phenolic compounds have been described that affect depigmentation either temporarily (such as hydroquinone) or in
a permanent fashion (including MBEH) (184). As treatments for vitiligo are largely ineffective, and patients with advanced depigmentation of their skin sometimes prefer an even skin tone, MBEH is included as the active ingredient of monobenzone cream, used to depigment the remainder of the skin over the course of several weeks to months (186).

The working mechanism of MBEH and other depigmenting agents is less well understood. Permanent depigmentation of the skin observed in most patients does suggest that the drug is capable of selectively eliminating melanocytes from the epidermis. Recent data showing selective cytotoxicity of MBEH towards melanocytes and not keratinocytes support this viewpoint (chapter 2) (187). Its selective action towards melanocytes has been explained by structural homology to tyrosine, the natural substrate for melanogenesis. When combined with the rate limiting enzyme for melanogenesis, tyrosinase, MBEH is converted into a highly reactive quinone that is suggested to react with surrounding enzymatic compounds within the confines of the melanosomal organelle (115). As expression of melanosomal enzymes is restricted to cells of the melanocyte lineage, the above sequence of events can explain a selective cytotoxic effect of MBEH on melanocytes. It should be considered that different phenolic compounds can follow markedly different depigmentation strategies. In this respect, whereas MBEH is thought to bind tyrosinase, 4-tertiary butyl phenol appears to interact with TRP-1 (118, 163).

Quinone binding to melanosomal compounds occurs in a process described as haptenization, whereby the hosting compound undergoes slight structural alterations
Melanosomal enzyme modification is significant as TRP-2, MART-1, gp100 and tyrosinase itself are immunogenic molecules, and minor changes in amino acid composition or even haptenization have been shown to alter their recognition by T cells reactive with the native peptide (183). By increased affinity for the modified peptide, immune tolerance can be broken and improved reactivity will likely ensue. This process is significant for understanding the depigmentation process in vitiligo.

Upon studying the effects of MBEH exposure for melanocyte viability, it was found that sensitivity to MBEH exposure is not directly correlated with the expression of a single melanosomal compound. Rather, there was an inverse relationship noted for sensitivity to MBEH and generation of melanin within the cell. More intensely pigmented cells are thus increasingly resistant to the compound (chapter 2) (187). This can have implications for the treatment of ethnic skin with MBEH.

Besides an understanding of the direct effects of bleaching phenols on pigmented cells, any consequences for cell viability will indirectly affect immune responses to follow. In this regard, the markedly different effects of MBEH and 4-TBP on melanocytes manifest themselves in the induction of necrosis and apoptosis, respectively, in exposed cells (187). Both death pathways are thought to have markedly different consequences on dendritic cell activation and it is thus likely that 4-TBP and MBEH exposures have markedly different kinetics of depigmentation in an in vivo setting.

The mechanism of action of bleaching phenols becomes particularly important when considering the specificity for cells of the melanocytic lineage. Besides normal
epidermal and ocular melanocytes, cells potentially sensitive to the effects of MBEH and 4-TBP can thus include melanoma cells. Systemic toxicity however limits the application of bleaching phenols in a therapeutic setting. The toxicity of both MBEH and 4-TBP towards fibroblasts, for example, is significant (chapter 2) (163, 187). To nevertheless make use of the unique qualities of bleaching phenols towards the treatment of melanoma, two supportive factors are considered here. First, eliminating melanocytes from the skin will prevent precursor cells from converting to melanoma cells by malignant transformation. Thus this process may be effective as a chemical alternative to elective surgery for patients familial melanoma, or to prevent recurrences in patients following surgical removal of a primary tumor. Second, cell death among a portion of melanocytes in the skin may elicit an immune response that carries over to an effective anti-tumor response by the recognition of antigens shared between melanocytes and melanoma cells (153, 188-189). Familial melanoma is a condition affecting approximately 10% of melanoma cases, or an estimated 6000 newly diagnosed patients per year in the United States alone (190). If induction of vitiligo can spare patients from agonizing returning visits to the dermatologist to check for newly arising dysplastic nevi, treatment with bleaching phenols may be a more effective and permanent way to address familial disease.

To study the potential of prophylactic treatment using bleaching phenols for familial melanoma, it is necessary to first identify the in vivo effects of the agents under study in an in vivo setting, in particular to address the anticipated involvement of an
immune response. Here, the effects of bleaching agents 4-TBP and MBEH were studied initially in normal human skin, in order to quantify Langerhans cell emigration in response to either agent. Organotypic cultures of human skin are ideal for these studies, as emigration can be studied without new influx of immune cells (170). Since Langerhans cells migrate upon antigen specific stimulation, the same approach has been followed to distinguish allergenic compounds from skin irritants (191-192). This same factor limits the use of skin cultures to assess T cell influx, and these studies were executed in mouse models instead. To this end, bleaching agents were applied to the skin of wildtype mice as well as a transgenic model designed to express melanocytes in the epidermal compartment of the skin. In the k14-SCF mouse, stem cell factor is expressed under the k14 promoter within keratinocytes to support melanocyte maintenance within the skin environment, which is important to follow topical application of the bleaching agents (193). Depigmentation was quantified in both models and T cell influx, immigration of macrophages and cytokine expressions were measured in the skin. To understand whether these agents can be used to target melanoma cells, the sensitivity of human melanoma cells were quantified by in vitro cytotoxic assays. Taken together, these data shed important light on the prophylactic potential of bleaching agents for the treatment of familial melanoma.
Materials and Methods

Mouse models

Two strains of mice, C57BL/6J from Jackson Labs (Bar Harbor, ME) and k14-SCF transgenic mice (193), were used in the depigmentation experiments. In the k14-SCF mice, mouse stem cell factor is expressed under the k14 promoter which maintains melanocytes within the epidermis, thus rendering k14-SCF mice a close mimic of human skin. Group sizes were 5 per group for C57BL/6J mice and 4 per group for k14-SCF mice unless stated otherwise. All experiments were approved by Loyola University Medical Center's Institutional Animal Care and Use Committee.

Preparation of bleaching agents and treatment

4-TBP (Sigma-Aldrich, St Louis, MO) was prepared as a stock solution of 3M in 70% ethanol. MBEH (Sigma) was dissolved in 20% dimethyl sulfoxide (Sigma) and mixed with 70% ethanol for a stock concentration of 3M. The use of a vehicle control refers to the use of 20% dimethyl sulfoxide in 70% ethanol. The chemicals were stored as a stock at -20°C until further use. Before necessary treatments, chemicals were mixed with Eucerin calming cream (Beierdorf, Wilton, CT) to a final concentration of 1M or 1.5M.

Mice were prepared for depigmentation treatment by biweekly ventral hair removal with Nair (Church and Dwight Co., Princeton, NJ). C57BL/6 mice were topically treated with 100µl of 1.5 M 4-TBP or MBEH applied to a 3 cm² area of Naired skin on alternate days for 6 weeks. The k14-SCF mice were similarly treated with 100µl
of 1M concentration of 4-TBP or MBEH for 14 weeks. Mice were anesthetized during
hair removal and bleaching phenol treatments using an isofluorane gas chamber
(Euthanex Corp, Palmer, PA).

**Organotypic culture of treated human skin**

Organotypic culture was performed as described previously (170). In brief, 4mm
biopsies were punched from otherwise discarded foreskin tissue obtained after routine
circumcision. Explants were cultured in DMEM containing 10% inactivated normal
human serum at the air–liquid interphase with epidermis facing up in 12-well transwell
plates with a 0.4 µM pore size (Corning, Teterboro, NJ). After topical treatment with 5 µl
of 250 mM of 4-TBP or MBEH daily for 3 days, explants were embedded in optimal
cutting temperature compound (OCT) (Sakura Finetek USA, Torrance, CA), snap-frozen
and maintained at -80 °C 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.

**Cell culture**

Mouse melanocyte cultures were initiated from untreated k14-SCF mice skin
samples. After euthanasia, mouse skin samples were homogenized O/N in an enzymatic
cocktail (thermolysin 0.05mg/ml, collagenase 1mg/ml, trypsin 0.1mg/ml, DNase
0.01mg/ml) prepared in DMEM (Media-Tech, Manassas, VA). Cells were cultured in
Ham's F-12 medium (Media-Tech, Herndon, VA) with 10% heat-inactivated fetal bovine
serum (Gemini Bio-products, West Sacramento, CA), 2mM glutamine (Media Tech),
100IU/ml penicillin, 100µg/ml streptomycin 100µg/ml amphotericin (Media Tech),
0.1mM IBMX (Sigma), 10ng/ml 12-O-tetradecanoyl phorbol-13-acetate (Sigma), and
0.03% bovine pituitary extract (Invitrogen). B16F10 mouse melanoma cells were
maintained in DMEM (Media-Tech) with 10% heat-inactivated fetal bovine serum
(Gemini Bio-products), 100IU/ml penicillin, 100µg/ml streptomycin and 100µg/ml
amphotericin (Media-Tech).

Evaluating depigmentation

Depigmentation was evaluated by flatbed scanning of the ventral side of the mice
under anesthesia, and subsequent image analysis using Adobe Photoshop software
(Adobe Systems Inc., San Jose, CA) as described previously (194). Depigmentation
experiments were repeated thrice and a representative experiment was shown. Briefly,
depigmentation is evaluated by quantifying mean luminosity of inverted images for the
treated area, comparing the data to untreated mice. Statistical analysis of data was
performed by comparing relevant groups by Student's T-test using Excel software.

Immunohistology

Biopsies of treated mouse skin, treated human skin explants, or mouse tumor
tissue were embedded in OCT compound (Sakura Finetek USA, Torrance, CA), snap-
frozen on dry ice. Eight µm cryostat sections were fixed in cold acetone and stored at
-20 °C until use.
Human explant sections were stained with antibody CD207 to Langerin (mouse monoclonal: Immunotech, Marselle, France). Vehicle application was used as a negative control. Recruitment of Langerhans cells was evaluated in 3 human skin biopsies per group. Mouse skin sections were stained with biotinylated hamster antibody 145-2C11 to CD3ε antibody (Pharmingen, San Diego, CA, or goat T-16 ab to CD68 (Santa Cruz Biotechnology Inc, Santa Cruz, CA). Peroxidase labeled anti-mouse or anti-goat antisera or peroxidase labeled streptavidin (Southern Biotechnologies, Birmingham, Alabama) was used in the second step and color was developed using aminoethyl carbazole (AEC) as a substrate (Sigma) as described earlier (144). Quantifications were performed in 2 serial sections from tissues of 4 mice/group. Stained cells were counted within the epidermis for CD3εT and Langerin + Langerhans cells to a depth of twice or four times the epidermal thickness in the dermis to quantify Langerin+Langerhans cells and CD68+ macrophages respectively. Epidermal length or area was calculated using Adobe Photoshop CS4 software (Adobe Systems Inc).

**Cytotoxicity Assay**

Cell viability was measured by MTT assays (Bioassay system, Hayward, CA) according to manufacturer’s instructions. In brief, 20,000 cells/well were plated in triplicate wells of a 96-well plate to attach overnight. Cells were either treated with vehicle alone or with 250, 500 or 900μM of 4-TBP or MBEH for 24 hours. 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 mitochondria of living cells. The formazan crystals
formed were solubilized in buffer and the wavelength was read in a reader at 562 nm (BMG labtech inc, Durham, NC). Cell viability was calculated as a percentage of absorbance of the samples relative to vehicle treated control.

**Evaluating DC maturation**

Bone marrow-derived monocytes were prepared by culturing murine bone marrow cells using protocols modified from Boudreau *et al* (195). Briefly, bone marrow cells were flushed aseptically from the femurs and tibia of mice. Monocytes were enriched using the EasySep mouse monocyte enrichment kit (Vancouver, BC) according to the manufacturer’s instructions. To generate DC, the monocytes were grown in RPMI (Mediatech Inc., Manassas, VA) supplemented with 10% FBS and 100 U/ml penicillin, 100 µg/ml streptomycin, 50 ng/ml murine GM-CSF (Peprotech, Rocky Hill, NJ) and 12.5 ng/ml murine IL-4 (Peprotech). Cells were plated at 2x10⁶ cells per 5 ml of media in Teflon containers (Savillex, Minnetonka, MN) to prevent adherence. Half of the media was replaced every other day for seven days. On day seven, 125 µM of 4-TBP, 63µM or 125 µM of MBEH, or 1µg/ml lipopolysaccharide (LPS) (Sigma) was added to the cells. After 24 h, cells were gently spun and stained with Hamster anti-mouse CD11c, CD80, CD83, CD86, and MHCIIm labeled with V450, APC, PE, PE-Cy and FITC fluorochromes respectively (BD Biosciences, Sparks MD). Initial gating was performed on live non-debris singlets, with subsequent gating towards CD11c cells using FACS LSR-II equipment (BD Biosciences).

**Real time PCR**
Cytokine profiling was performed in treated mouse skin in remaining subcutaneous tumor tissue. Sample sizes were 5 per group for skin samples and 3 for remaining tumor tissue obtained from vehicle and 1.5M MBEH-treated animals. RNA was isolated from tissue sections using TRIzol reagent (Invitrogen, Carlsbad, CA) and further purified using an RNeasy mini kit (Qiagen, Valencia, CA). Two μg of RNA was reverse-transcribed with superscript III (Invitrogen) and 10% of resultant cDNA was included in the real time PCR reaction. Real time PCR was performed using Quantitect SYBR green master mix and samples were run in triplicate and amplified at 95°C for 10 minutes, 95°C for 15 seconds, 55°C for 30S, 72°C for 30S for 50 cycles. Standard mouse primer sets and probes for IFNγ, IL-2, IL12b, IL17a, TNFα, IL-10, IL-4, and GAPDH were purchased from Qiagen. Relative fold change in gene expression was calculated using the ΔΔCt method after normalizing with vehicle treatment (196-197). Experiments were repeated thrice. Representative data from a single experiment is shown.

RESULTS

Evaluating sensitivity of melanoma cells to bleaching phenols

Viable melanoma cells (624.38, 888 and A375) and melanocytes were identified using green fluorescent marker calcein AM after treatment with 900 μM of 4-TBP or MBEH. A substantial reduction in viability was observed in melanocytes with both 4-TBP and MBEH treatment, while no changes were observed in melanoma cells (Fig. 12a). Representative fluorescent images are shown in Fig.12a. The sensitivity of melanoma and melanocytes were then quantified using MTT assays after 250 and 500
μM treatment of 4-TBP or MBEH for 24 hours. Both 4-TBP and MBEH induced >65% of cell death in melanocytes, while melanoma cells maintained >60% viability upon treatment (Fig.12b). To identify whether resistance of melanoma cells to bleaching agents was due to loss of melanocyte markers, the basal level of melanocyte markers was evaluated by intercellular staining of melanoma and pooled melanocyte cultures and analyzed by FACS. Fig.12c indicates expression levels of tyrosinase, TRP-1 and TRP-2 corresponding to the relative sensitivity towards either agent.
Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)

Viability (%)
Fig. 12 Sensitivity of melanoma cells to 4-TBP and MBEH

a) Melanoma cultures exposed to 900 µM of 4-TBP or MBEH for 1 hour and treated with Calcein AM to visualize live cells. b) Sensitivity of human melanoma cell lines A375, M14 (amelanotic), 888, 624.38 (melanotic) was compared with melanocyte cultures (pooled dark and light melanocytes) to 250 and 500 µM of 4-TBP or MBEH for 24 hours.
Viability was measured by MTT assays. c) Basal melanocyte marker expression in melanoma cells and melanocytes were compared with their respective viability upon 500 µM treatment with 4-TBP or MBEH for 24 hours. Results of studies comparing basal marker expression with relative sensitivity suggest potential involvement of tyrosinase and TRP-1 in sensitizing melanoma cells upon bleaching agent treatments.
Eliciting immune responses by 4-TBP and MBEH in skin explants

Langerhans cell abundance and migration were monitored as a measure of the elicitation phase of an immune response in human explant cultures treated with 250mM of 4-TBP or MBEH for 3 days (Fig.13). Representative staining for each treatment is shown in Fig.13a. Although the total number of Langerin+ cells in the epidermis remained constant (Fig.13b), agents 4-TBP and MBEH respectively induced a 2.4 and 2.9 fold increase in migration of Langerhans cells towards the basal layer of the epidermis in comparison to vehicle treatment alone (p<0.05)(Fig.13c). In addition, MBEH and not 4-TBP treatment caused a 2.0 fold increased migration into the dermis over control treatment (p<0.01) (Fig.11d). These data support that the immune activation particularly upon MBEH treatment.
Langerhans cell/mm

Mean± SD

Control 4-TBP MBEH

Langerin number/epidermal length (mm)

Control 4-TBP MBEH
Fig. 14 Eliciting immune responses by 4-TBP and MBEH in human skin explants

Explant skin cultures were treated with 250 mM of 4-TBP or MBEH for 3 days and stained for the presence of Langerhans cells. Vehicle treatments alone served as controls. (a) Representative image of Langerin staining for each treatment is shown. (b) Quantification of total Langerhans cells in the epidermis (c) Quantification of Langerhans cells in the basal layer represented as number of cells/mm epidermal length. (d) Quantification of Langerhans cells in the reticular dermis expressed as number of cells/mm². Mean and SE were calculated and statistical significance was evaluated by Students’ t-test (n=3) for epidermal measurements and (n=4) for dermal measurements. ** indicates p<0.05. A 2.4- and 2.9-fold increase in migration of Langerhans cells towards the basal layer of the epidermis was observed upon 4-TBP and MBEH treatment respectively over control, while a 2.0-fold increase in migration of LC into the dermis over control was observed only upon MBEH treatment.
Effect of phenolic agents on dendritic cell maturation

Immature myeloid dendritic cells derived from bone marrow precursors were treated with 63 or 125 μM of MBEH, 125 μM of 4-TBP for 24 hours. Cells stained for activation markers were quantified in live CD11c positive cells (Fig. 14). While no activation was observed upon 4-TBP treatment, 1.6-, 1.3-, 1.1- and 1.1- fold activation for CD80, CD83, CD86 and MHCII respectively was observed upon MBEH treatment. Such limited activation and maturation may contribute to the migratory behavior of DC subsets in response to MBEH, but not 4TBP treatment.
Fig. 14 Effect of phenolic agents on dendritic cell maturation

Immature dendritic cells derived from bone marrow precursors were treated with 125μM of 4-TBP, 63 μM or 125μM of MBEH for 24 hours. Live Cd11c+cells were then stained for dendritic cell activation markers. Vehicle treatment served as a negative control while LPS treatment served as a positive control. Experiments were repeated thrice and data from a representative experiment are shown in Fig. 12. While no activation was observed upon 4-TBP treatment, 1.6-, 1.3-, 1.1- and 1.1- fold activation for CD80, CD83, CD86 and MHCII respectively was observed upon MBEH treatment.
Topical application of bleaching agents in wild type mice

C57BL/6 mice were shaved biweekly to remove the ventral pelage and topically treated with 100ul of 1.5 M MBEH or 4-TBP on alternating days for 6 weeks. Two weeks after cessation of treatment, mice were scanned on a flat bed scanner and changes in pigmentation of the pelage in the treatment area were measured by image analysis. MBEH treatment induced a 20.5% reduction in pigmentation (p<0.0001), while 4-TBP treatment induced 2.8% depigmentation (p<0.01) relative to vehicle treatment (Fig. 15b). Scanned mice images were shown in Fig.15a. These data demonstrate that MBEH penetrates the mouse skin at least to the follicular level.
Fig. 15 Topical application of bleaching agents in wild type mice

C57BL/6 mice were shaved biweekly to remove ventral hair and topically treated with 100μl of 1.5M MBEH or 4-TBP on alternating days for 6 weeks. Two weeks after cessation of treatment, mice were scanned on a flat bed scanner and changes in depigmentation quantified. (a) Scanned images of C57BL/6 mice for each treatment immediately following the cessation of the experiment. (b) Change in percent
depigmentation with respect to the vehicle treatment. Mean and SE were calculated and statistical significance was evaluated by the Student T test (n=5). ‘**’ indicates p<0.01: ‘***’ indicates p<0.0001. MBEH treatment induced a 20.5% reduction in pigmentation, while 4-TBP treatment induced 2.8% depigmentation.
**Effect of bleaching agents on epidermally pigmented k14-SCF mice**

Shaven ventral skin of k14-SCF mice was topically treated with 1M concentration of 4-TBP or MBEH on alternating days for 14 weeks. Changes in the pigmentation levels upon treatment with vehicle, 4-TBP or MBEH were shown among the regrown pelage of k14-SCF mice in Fig.16a and 16b. The percent change in pigmentation was quantified relative to vehicle treatment. 4-TBP treatment had no significant change in pigmentation level in the regrown hair (Fig. 16b). However, MBEH caused 12.9% (p<0.0001) depigmentation in hair when compared to vehicle treatment (Fig. 16b). This dataset demonstrates that MBEH has a depigmenting effect on hair follicles in mice.
**Fig. 16 Effect of bleaching agents on epidermally pigmented k14-SCF mice**

Shaven ventral skin of k14-SCF mice was topically treated with 1M concentration of 4-TBP or MBEH on alternating days for 14 weeks. Changes in pigmentation were measured among regrown hair after the final treatment. (a) Scanned images of the k14-SCF mice after regrowth of their hair. (b) Bar graphs represent percent depigmentation levels of regrown hair with respect to vehicle treatment. Mean and SE were calculated and statistical significance was evaluated by Student’s t-test (n=4). ‘***’ indicates
p<0.0001. MBEH treatment caused 12.9% depigmentation in the hair, while no depigmentation was observed upon 4-TBP treatment.
**Immune cell influx in response to MBEH versus 4-TBP**

T cell and macrophage influx to the skin was quantified by staining for CD3^+ and CD68^+ cells in epidermal and dermal skin regions respectively treated with 1M concentration of either bleaching agents in k14-SCF mice for 14 weeks. MBEH treatment induced a 2.0-fold increase in T cell infiltration (p<0.05), whereas 4-TBP treatment resulted in a 0.5-fold reduction in the T-cell number when compared with control treated skin (not significant) as shown in Fig. 17a. A similar trend was observed upon macrophage infiltration to the dermal area of treated k14-SCF skin. MBEH treatment induced a 2.4-fold increase in CD68^+ cells (p<0.01), while 4-TBP treatment did not result in a significant increase in macrophage infiltration compared with vehicle-treated skin (Fig. 17b). This further supports the concept that MBEH activates an immune response while no difference was observed upon 4-TBP treatment.
**Fig.17 Immune cell influx in response to MBEH versus 4-TBP**

T cell and macrophage influx to the skin was quantified by staining for CD3+ and CD68+ cells in epidermal and dermal skin regions treated with 1M concentration of either bleaching agents in k14-SCF mice for 14 weeks. (a) Influx of CD3+ cells in the epidermal region of the mouse skin was quantified in 2 independent experiments and expressed as number of stained cells/mm epidermal length. (n=6 for 4-TBP and MBEH treatment; n=4 for vehicle treatment) (b) Influx of macrophages in to the dermal regions of k14-SCF mice skin samples were quantified and expressed as the number of positive cells/dermal area (mm2). Mean and SE were calculated and statistical significance was evaluated by Student one-tailed T test. ‘*’ indicates p<0.05, ‘**’ indicates p<0.01. MBEH treatment led to a 2 fold and 2.4 fold increase in the influx of CD3+ and CD68+ respectively, while no significant changes was observed upon 4-TBP treatment.
Cytokine profiling in MBEH treated mouse skin

Cytokine environment was studied in frozen C57BL/6 mice skin after treatment with vehicle or 1.5M MBEH for 6 weeks (Fig. 18). Increases in the relative transcript levels of TNF-\(\alpha\), IFN-\(\gamma\) and IL-2 were observed at a 2.8, 2.4, and 2.4 fold, respectively over vehicle treatment. Expression levels of IL-10, IL-4, IL-12 and IL-17 were not altered, thus supporting a Th1 mediated immune response.
Fig. 18 Cytokine profiling in C57BL/6 mice skin treated with MBEH

Transcript levels of pro and anti-inflammatory cytokines evaluated by qRT-PCR in mice skin samples treated with vehicle or 1.5 M MBEH for 6 weeks. Relative fold change in the expression levels are shown upon normalization with vehicle treatment. Mean and standard deviation were calculated from 3 replicates. Experiments were repeated thrice and data from a representative experiment are shown. A Th1 mediated immune response was observed upon MBEH treatment.
Discussion

Vitiligo patients with advanced disease can opt for depigmentation therapy, wherein topical application of bleaching agents is used to remove any remaining pigmentation (184). The data presented here demonstrate that depigmentation in response to MBEH can be reproduced in an *in vivo* mouse model. In accordance with our earlier studies, the increased sensitivity of melanocytes to MBEH as compared to 4-TBP *in vitro* (187) is accompanied by actual greater treatment efficacy *in vivo*. Contrary to expectation, depigmentation of the pelage occurred more readily than depigmentation of the skin. This may in part be a consequence of the densely populated and heavily melanized epidermis of k14-SCF mice, as we have previously reported that melanin may protect against the cytotoxic effects of MBEH (187).

Such data are of particular importance as the elimination of melanocytes from the skin and hair follicles can theoretically serve as an alternative to ‘elective surgery’ offered for patient with mutations in tumor-associated genes (142, 198). For example, such elective surgery is offered to patients with mutations in BRCA-1 or BRCA-2 where the odds of developing breast- or ovarian cancer can increase from ~1% to virtually 100% (198). As preventive surgery is out of the question for familial melanoma patients, the concept of chemoprevention proposed here can offer a much needed alternative for patients with mutations in INK4a/ARF or CDK4 genes (14, 199). This idea is further strengthened by the fact that melanoma cells are significantly resistant to either agent in
comparison with melanocytes, thus making them potential chemopreventive agents for familial melanoma.

As we have previously established, sensitivity to MBEH is specific for melanocytes in the epidermis, but other cell types can display similar sensitivity to both MBEH and 4-TBP, including fibroblasts (187). This precludes the use of bleaching phenols in systemic applications, which is desirable in a therapeutic setting for melanoma. Indeed, several authors have suggested the use of bleaching agents in the treatment of melanoma(117).

It is an intriguing concept that bleaching agents can serve as a prodrug to be selectively converted by enzymes uniquely expressed by melanocytes and melanoma cells to generate toxic quinones. In fact, it has been proposed that resulting quinones may in turn convert molecules in close proximity into increasingly immunogenic moieties according to the ‘haptenization theory’ (115, 118). In this regard, the accelerated depigmentation in response to MBEH in the order of magnitude observed here further suggests that part of the depigmentation effects observed in vivo may actually be indirect. We postulate that the necrotic pathway of cell death induced by MBEH as opposed to apoptosis following exposure to 4-TBP may differentially affect processing and presentation of melanocyte antigens and selectively lead to an immune response after MBEH exposure (167-168, 187). Indeed, the data presented here strongly support elicitation of an immune response in response to MBEH that is not found after 4-TBP application. First, we observed Langerhans cell emigration from the epidermis of human
skin in response to MBEH, whereas after 4-TBP application these cells were merely found in basal position, thus remaining within the epidermis. It is likely that antigens processed by intra-epidermal Langerhans cells will not be presented to T cells in draining lymph nodes, thus preventing an influx of T cells to the skin. Such investigations required moving from ‘ex vivo’ model in human tissue to ‘in vivo’ studies by engaging C57BL/6 or k14-SCF mice with epidermal melanocytes. In these in vivo studies, it was observed that both macrophages and T cells primarily driven by Th1 cytokines infiltrate the skin in response to MBEH, but not 4-TBP. A combination of infiltrating macrophages and T cells is similarly observed in vitiligo and other autoimmune diseases and this combined effect might be responsible for the removal of target cells under stress (158, 200-201).

Thus, the data presented here demonstrate that MBEH targets the melanocytes as well activate a Th1 mediated immune response, thus acting as a potential agent for preventive treatment for people with increased predisposition towards melanoma development.
**Fig. 19 Summary of Chapter 3**

Topical application of MBEH induces significant depigmentation in mouse models. Further, application of MBEH and not 4-TBP cause emigration of Langerhans cell in human skin and a Th1 mediated cytokine environment accompanied by an infiltration of macrophages and T cells in mice skin.
CHAPTER IV

IN VIVO ASSESSMENT OF ANTI-TUMOR POTENTIAL OF MBEH

Abstract

Ideally, a familial melanoma mouse model to be used for studying the chemo preventive effects of topically applied bleaching agents should have melanocytes in the epidermis with genetic alterations that predispose them to malignant transformation. However, in wild type mice, melanocytes are located strictly in hair follicles and the animals lack epidermal pigmentation. Here an attempt was made to generate a suitable mouse model for our studies, reflecting the chemo preventive potential of bleaching agents for familial melanoma treatment. This can be achieved by crossing mice with expression of activated H-RAS $G^{12V}$ (encoded on Y chromosome) driven by a tyrosinase promoter on an Ink4a/Arf deficient background, and k14-SCF transgenic mice with epidermal pigmentation in their skin due to transgenic expression of stem cell factor (SCF) under the keratin 14 promoter. Tumors generated among pigmented, male Ink4a/Arf $-/-$ offspring were assessed for the presence of melanoma markers by FACS, immunohistochemistry and Western blot techniques. Generation of non pigmented cutaneous tumors among male offspring of the H-RAS $G^{12V}$ Ink4a/ARF $-/-$ k14-SCF mice suggested the absence of melanoma tumors. Indeed, tumors developing in parental male H-RAS $G^{12V}$ Ink4a/ARF $-/-$ mice similarly lacked expression of melanoma markers
suggested that activated RAS expression may not be driven by the tyrosinase promoter. Thus, the tumor preventive effect of bleaching agent MBEH was instead identified by pretreating k14-SCF mice and challenging them with B16 melanoma tumors. Pretreatment of k14-SCF mice by MBEH significantly retarded tumor growth in mice challenged with B16 melanoma cells, despite a clear insensitivity of human and mouse melanoma cells to MBEH induced cytotoxicity. An abundance of cytotoxic T cells accompanied by an increased expression of Th1 and Th17 cytokines observed in remaining tumor tissues further emphasize immune mediated retardation of melanoma growth. This protective immune effect is likely due to generation of antigenic substrate by dying epidermal melanocytes. These data support the use of MBEH as a prophylactic treatment for melanoma.

Introduction

Challenging wild type mice with B16 melanoma cells is the most widely used in vivo model system to study the efficacy of proposed treatments for melanoma. Identification of numerous genetic abnormalities in melanoma patients and a better understanding of signaling mechanisms involved in melanoma genesis, led to the development of several spontaneous melanoma mouse models to study the involvement of the genes products of interest. Initial observations by Kamb et al that the CDKN2A gene was either mutated, hyper methylated or deleted in more than 75% of human melanoma cell lines led to the development of knockout mouse models for p16\textsuperscript{Ink4a} and p14\textsuperscript{Arf} (6). Surprisingly, complete knockout of Ink4a/Arf led to spontaneous tumors other than melanomas (32). However, approximately 50% of mice with complete loss of Ink4a
but with one copy of Arf generated melanomas upon UV or DMBA exposure (202). These results suggest that loss of Ink4a is essential for tumorigenesis. However, maintaining Arf in the heterozygous form might drive melanoma formation upon carcinogen exposure. However, it is not clear how maintaining a single copy of Arf would help in melanoma formation since Arf was shown to be present in the melanomas after carcinogen exposure.(202).

As a variant to this model, Dr Chin and colleagues have since generated Ink4a/Arf knockout mice expressing activated HRAS under the tyrosinase promoter (203). It should be noted that the Tyr:HRAS transgene is present on the Y chromosome thus rendering only males susceptible to melanoma. These mice are generated on an unpigmented (albino) background due to a common nonsense mutation truncating the tyrosinase protein. In the absence of external stimuli these mice develop unpigmented tumors in approximately 16-20 weeks.

Since our primary interest is to understand the effects of topical depimentation, it is important to note that in the mouse, melanocytes are found in hair follicles rather than in the epidermis (204). Mouse melanocytes can be re-directed to the epidermis during development by epidermal expression of growth factors that support the maintenance of melanocytes within this compartment. Existing models with epidermal melanocytes include HGF and SCF transgenic mice. HGF transgenic mice were generated on an albino background where mice do not express functional tyrosinase, and resulting melanocytes are detected both in the dermis and in the epidermis (205). This model was
previously crossed with the Ink4a/ARF -/- model, and resulting mice have been instrumental in demonstrating a link between melanoma genesis and UV exposure in the absence of melanin (206). To study the chemopreventive effects of topical depigmenting agents to familial melanoma, the expression of functional tyrosinase by melanocytes is essential to recapitulate the conversion of bleaching phenol substrate into cytotoxic quinones by tyrosinase exclusively in melanocytes (155). Pigmented, tyrosinase expressing melanocytes are also a source of tyrosinase-derived antigenic peptides that can enhance anti-tumor immunity (207). Mice that express membrane bound but not secretory SCF under the k14 promoter carry melanocytes in the epidermis in the absence of mast cell hyperproliferation (193).

In this chapter, attempts are described to generate the optimal model for testing the prophylactic potential of topical bleaching treatment for familial melanoma. Initially, the spontaneous cutaneous tumors among males mouse model (SCAM) mice model was generated by crossing Tyr(Y) -HRAS Ink4a/Arf-/- Tg mice with the k14- SCF transgenic model to generate mice that would ultimately develop pigmented melanoma in the epidermal compartment.

In previous chapters, it was demonstrated that 4-TBP and MBEH are toxic to melanocytes in comparison with keratinocytes (chapter2). In addition, 4-TBP and MBEH are shown to activate apoptotic and necrotic death of melanocytes (chapter 2). Since the mode of cell death plays an important role in the resultant immune response, it was hypothesized that MBEH might activate an immune response. *Ex vivo* studies in treated
explants clearly indicated that MBEH might activate an immune response as it mediated emigration of Langerhans cells from the epidermis (chapter 3). In addition, MBEH was shown to induce potent depigmenting effects in C57BL/6 and k14SCF Tg mice. Finally, the topical application of MBEH in mice led to increased production of pro inflammatory cytokines and increased skin infiltration by T cells and macrophages (chapter 3). Thus, MBEH was identified as a potential candidate that can activate a two-tiered response- removing the melanocytes and activating an immune response.

In this chapter, the anti-tumor immune response mediated by MBEH is described by assessing the sensitivity of B16 melanoma cells to the cytotoxic effects of MBEH first, followed by pre-treating k14-SCF mice and challenging them with melanoma cells subcutaneously. Immune monitoring is performed on remaining tumors in pretreated and untreated mice, both by qRT-PCR to quantify cytokine transcripts and indentify the type of ongoing immune response, and by immunohistology to evaluate cytotoxic T cell infiltration.

**Materials and methods**

**Preparation of bleaching agents for in vivo treatment**

4-TBP was prepared as a stock solution of 3M in 70% ethanol. MBEH was dissolved in 20% dimethyl sulfoxide (Sigma, ST Louis, Mo ) and mixed with 70% ethanol for a stock concentration of 3M. The use of a vehicle control refers to the use of 20% dimethyl sulfoxide in 70% ethanol. The chemicals were stored as a stock at -20°C until further use. Before necessary treatments chemicals were mixed with Eucerin
calming cream (Beierdorf, Wilton, CT) to a final concentration of 1 or 1.5M with a corresponding weight of 20 or 30 mg per 100μl respectively for MBEH, 15 and 20 mg per 100μl respectively for 4-TBP.

**Generation of SCAM model**

The spontaneous cutaneous tumors among males mouse model (SCAM) was generated by crossing k14-SCF mice (193) and Tyr H- Ras^{G12V} (only in males) Ink4a/Arf/-/- mice (199). For convenience, Tyr H- Ras^{G12V} (only in males) Ink4a/Arf/-/- mice will henceforth be referred as (1) and k14- SCF transgenic mice as (2).

**Step 1**

Since the constitutive active RAS is present only in the males of (1), males from (1) were crossed with females from (2). The phenotypes for both mouse strains are listed below in Table1. The offspring from this cross are 100% heterozygous for the Ink4a/Arf deletion and k14-SCF pigmentation, with all males expected to express active RAS under the tyrosinase promoter.

**Table 1: Parental strains used**

<table>
<thead>
<tr>
<th>Y-TYR RAS Ink4a/Arf -/-</th>
<th>K14-SCF mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ink4a/Arf (-/-) (aa)</td>
<td>Ink4a/Arf (+/+)(AA)</td>
</tr>
<tr>
<td>Tyr-RAS (-/+)(b^xB^y)</td>
<td>Tyr-RAS (-/-) (bb)</td>
</tr>
<tr>
<td>K14-SCF (-/-) (cc)</td>
<td>K14-SCF (+/+)(CC)</td>
</tr>
<tr>
<td>Wild type tyrosinase (-/-)(dd)</td>
<td>Wild type tyrosinase (+/+)(DD)</td>
</tr>
</tbody>
</table>

**Table 1**: The Ink4a/Arf -/- mice have complete loss of Ink4a/Arf gene (aa) and have active RAS under Y chromosome (b^xB^y), these mice also have mutated non functional
tyrosinase gene (cc). The k14-SCF mice have wild type Ink4a/Arf (AA) and tyrosinase genes (DD), with an SCF transgene under the k14 promoter (CC).

**Step-2** The F1 generation (Aab\(^b\)CCdD-female) was backcrossed with a male parent (aab\(^b\)B\(^c\)ccdd) and vice versa. This backcross helped to generate homozygous knockouts of Ink4a/Arf. Among the offspring, 16 possible outcomes can be obtained of which 12 were excluded by visual screening as they are either albino or do not express melanocytes in the epidermis. The remaining genotypes were heterozygous for pigmentation and epidermal melanocytes with all males exclusively expressing activated RAS.

**Punnett square-Back cross**
Aab\(^b\)CcDd (female offspring) X aaB\(^b\)ccdd (male parent)

**Phenotype of interest from backcross:**
- **aa** - INK4a/ARF homozygous
- **B\(^b\)** - Constitutive active RAS only in males
- **Cc** - k-14 SCF heterozygous
- **Dd** - wild type Tyrosinase.
Table 2: Back cross of female

<table>
<thead>
<tr>
<th>Parental strain</th>
<th>aB×cd</th>
<th>ab×cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab×Cd</td>
<td>AaB×b×CcDd (male)</td>
<td>Aab×b×CcDd (female)</td>
</tr>
<tr>
<td>Ab×Cd</td>
<td>AaB×b×Ccdd (albino)</td>
<td>Aab×b×ccDd (albino)</td>
</tr>
<tr>
<td>Ab×cD</td>
<td>AaB×b×ccDd (no-pig)</td>
<td>Aab×b×ccdd (no-pig)</td>
</tr>
<tr>
<td>Ab×cd</td>
<td>AaB×b×ccdd (both)</td>
<td>Aab×b×ccdd (both)</td>
</tr>
<tr>
<td>ab×CD</td>
<td><strong>aaB×b×CcDd (male)</strong></td>
<td><strong>aab×b×CcDd (female)</strong></td>
</tr>
<tr>
<td>ab×Cd</td>
<td>aaB×b×Ccdd (albino)</td>
<td>aab×b×ccdd (albino)</td>
</tr>
<tr>
<td>ab×cD</td>
<td>aaB×b×ccDd (no-pig)</td>
<td>aab×b×ccDd (no-pig)</td>
</tr>
<tr>
<td>ab×cd</td>
<td>aaB×b×ccdd (albino)</td>
<td>Aab×b×ccdd (albino)</td>
</tr>
</tbody>
</table>

**Table 2**: Punnett square for the backcross between the female offspring and male parent.

The highlighted offspring were included in the next step.

Homozygous loss of the Ink4a/Arf gene was assessed by PCR from the DNA obtained from the tail snips of selected male and female mice. Since the neomycin cassette was used as a selection marker for the knockout of Ink4a/Arf gene, presence of neomycin and absence of the Ink4a/Arf gene both confirmed the loss of Ink4a/Arf. In addition, the presence of human RAS observed among the male offspring was used to confirm the integration of the RAS transgene into the Y chromosome.

**Step 3**

Ink4a/Arf-/- and RAS positive males were crossed with Ink4a/Arf-/- RAS negative females, both with epidermal pigmentation. Male offspring from this cross were observed for the development of cutaneous melanoma in about 17-18 weeks. Since the albino Tyr
H-Ras $^{G12V}$ (in Y chromosome) Ink4a/Arf-/- mice was generated on an agouti background, the F1 generation has an agouti coat color.

**Genotyping**

DNA was isolated from the tail snips of the desired mice offspring for genotyping. The tail snips were incubated overnight at room temperature in tail salt buffer consisting of 1% SDS, 5mM EDTA, 10mM Tris (pH 8.0), 100mM NaCl, 200ug/ml of fresh proteinase K (Sigma, StLouis). Degraded protein was then precipitated by incubating at 4°C with the tail salts made up of 4.21 M NaCl, 0.63 M KCL, 10mM Tris (pH 8.0). The supernatant was mixed with 80% alcohol to precipitate the DNA. The DNA was resuspended in Tris EDTA buffer. Desired DNA segments were amplified in the presence of 50 mM MgCl$_2$, 10mM of dNTPs, 2.5 μl of 10X PCR buffer (Fermentas, Maryland), 0.5 μl Taq Polymerase (Fermentas, Maryland) and 20 μM of each primer. PCR amplification of target genes was performed at [95°C 20 seconds followed by 58°C 30 seconds and 72°C 1minute] 40 X. Amplification was completed by incubation at 72°C 10 minutes. The GAPDH primers and Ink4a/ARF primers used were derived from work published by Chin et al (199) while human RAS primers were published by Scherl et al (208) as shown in the table below.

To verify whether RAS is expressed under the tyrosinase promoter, forward primers were designed in the -270 to-80bp region of the tyrosinase promoter supposedly included in the original construct. This 270bp sequence 5’ to the tyrosinase gene is shown to be necessary and sufficient to maintain cell type-specific and developmentally
regulated expression of the tyrosinase gene (209). DNA from the tail of male Ink4a/Arf mice was amplified using PCR master mix (Fermentas) with an upstream primer binding within the tyrosinase promoter and a downstream primer to specifically amplify human Harvey RAS and not endogenous mouse RAS. Additional primers were designed to amplify human RAS and mouse tyrosinase as positive controls. Primers used are listed in the table.

Table 3 Primers used for genotyping in the generation/validation of SCAM model

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Neo-F</td>
<td>TGCTCCTGCGAGAAATGAATCCATCAGGC</td>
</tr>
<tr>
<td>Neo-R</td>
<td>CGCCAAGCTCTTCAGCAATATCACGGGAG</td>
</tr>
<tr>
<td>Ink4a-2F</td>
<td>GTGATGATGATGGGCAACGTTTC</td>
</tr>
<tr>
<td>Ink4a-2R</td>
<td>GGCGGTGTGGAGCTGAAGCTGAAG</td>
</tr>
<tr>
<td>Ink4a-3F</td>
<td>AGGGCCCTGGAACCTTCGCGGC</td>
</tr>
<tr>
<td>Ink4a-3R</td>
<td>GCTAGACACGCTAGCCTACG</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>ACCACAGTCCATGCCATCAC</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>TCCACCACCTGTTGGCTGTA</td>
</tr>
<tr>
<td>H- RAS-F</td>
<td>CAGGCCCCTGAGGAGCGAGGATG</td>
</tr>
<tr>
<td>H- RAS-R</td>
<td>ATGTACTGTGTCGCCGCATGCG</td>
</tr>
<tr>
<td>m TYR-F</td>
<td>CAATTAGTTACCTCACTATGAG</td>
</tr>
<tr>
<td>m TYR-R</td>
<td>GCCCATAGTGGTACTAATGATG</td>
</tr>
<tr>
<td>TYR- PRO-F1</td>
<td>TGTTTCATGACTTATGACAAG</td>
</tr>
<tr>
<td>TYR- PRO-F2</td>
<td>CAATTAGTTACCTCACTATGGGC</td>
</tr>
<tr>
<td>H-RAS-R2</td>
<td>GCTGGATGGTCACAGCGACTCTTG</td>
</tr>
<tr>
<td>H-RAS-R1</td>
<td>CTGTGATGCTGAATCGGGGTG</td>
</tr>
</tbody>
</table>

The Ink4a/Arf primers were designed in exons 2 and 3 of the CDKN2 gene, represented as Ink4a 2 and 3 respectively. The primers designed to amplify Human Harvey RAS,
neomycin, the tyrosinase promoter and GAPDH are represented as H-RAS, TYR-PRO, GAPDH respectively. The primer combinations and amplification length are provided in the table below.

**Table 4: Estimated product length for primers combinations**

<table>
<thead>
<tr>
<th>Primer combination</th>
<th>Amplification length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neo F+Neo-R</td>
<td>380</td>
</tr>
<tr>
<td>Ink4a-2F+2R</td>
<td>339</td>
</tr>
<tr>
<td>Ink4a 3F+3R</td>
<td>104</td>
</tr>
<tr>
<td>GAPDH F+R</td>
<td>425</td>
</tr>
<tr>
<td>H-RAS F+R</td>
<td>500</td>
</tr>
<tr>
<td>m-TYR PRO F+R</td>
<td>132</td>
</tr>
<tr>
<td>TYR- PRO-F1+H-Ras-R2</td>
<td>1368</td>
</tr>
<tr>
<td>TYR-2F+H-RAS-R1</td>
<td>658</td>
</tr>
<tr>
<td>TYR-PRO-1F+H-RAS-R1</td>
<td>774</td>
</tr>
</tbody>
</table>

**Cell culture**

Mouse melanocyte cultures were initiated from untreated k14-SCF mice skin samples. After euthanasia mouse skin samples were homogenized O/N in an enzymatic cocktail (thermolysin 0.05mg/ml, collagenase 1mg/ml, trypsin 0.1mg/ml, DNase 0.01mg/ml) (Roche, Indiana) prepared in DMEM (Media-Tech, Manassas, VA). Cells were cultured in Ham's F-12 medium (Media-Tech, Herndon, VA) with 10% heat-inactivated fetal bovine serum (Gemini Bio-products, West Sacramento, CA), 2mM glutamine (Media Tech), 100IU/ml penicillin, 100μg/ml streptomycin 100μg/ml amphotericin (Media Tech), 0.1mM IBMX (Sigma), 10ng/ml 12-O-tetradecanoyl phorbol-13-acetate (Sigma), and 0.03% bovine pituitary extract (InVitrogen).
B16F10 mouse melanoma cells were maintained in DMEM (Media-Tech) with 10% heat-inactivated fetal bovine serum (Gemini Bio-products), 100IU/ml penicillin, 100µg/ml streptomycin and 100µg/ml amphotericin (Media Tech).
The tumors isolated from male Tyr HRAS Ink4a/Arf-/- and SCAM mice were directly plated or digested in a cocktail of thermolysin, DNase, collagenase and trypsin overnight and plated in media containing DMEM (Media-Tech) with 10% heat-inactivated fetal bovine serum (Gemini Bio-products), 100IU/ml penicillin, 100µg/ml streptomycin and 100µg/ml amphotericin (Media Tech).

**Intracellular staining**

Mouse melanocytes and tumor cells from SCAM mice were fixed in 2% paraformaldehyde and permeabilized using saponin. The tumor cells and mouse melanocytes were stained with a cocktail of mouse monoclonal antibodies Ta99 to TRP-1 (Covance, Dedham, MA), T311 to tyrosinase (Neomarkers, Fremont, MA), M2-9E3 to MART-1 (Covance), HMB45 to gp100 (DAKO) at 4°C for 1 hour. Biotinylated rabbit anti mouse immunoglobins were as used as secondary antisera (Santa Cruz Biotechnology Inc., Santa Cruz, CA) followed by PE or APC labeled streptavidin. Fluorescence data were acquired with a BD FACScanto flow cytometer (Becton Dickinson) and analyzed with Flowjo software (Treestar).

**Western Blots**

Proteins isolated from B16F10 melanoma cells, SCAM tumor cells, mouse kidney cells and Tyr HRAS Ink4a/Arf-/- mice tumor cells were electrophoresed in a 10%
polyacrylamide gel and were transferred to a PVDF membrane (Millipore, Billerica, MA). The blots were incubated with a mouse monoclonal antibody Ta99 to TRP-1 (Covance, Dedham, MA). Peroxidase labeled, isotype specific goat antimouse serum was used in the second step. The red color was developed using aminoethyl carbazole as substrate (Sigma, St Louis).

**Polymerase chain reaction**

RNA from SCAM tumor cells and mouse melanocytes was isolated using TRIzol and was reverse-transcribed with superscript III (Invitrogen, CA). One μg of cDNA was amplified in presence of 50 mM MgCl₂, 10mM of dNTP, 2.5 μl of 10X PCR buffer (Fermentas, Maryland), 0.5 μl Taq Polymerase (Fermentas, Maryland) and 20 μM of each primer. PCR amplification of target genes was performed at [95°C 20 seconds] followed by [58°C 30 seconds, and 72°C 1 minute] 40 X. The reaction was completed at 72°C for 10 minutes. The primers used are stated below

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse gp100 F</td>
<td>ATGGGTGTCCAGAGAAGGAGCTTCCCTTCCC</td>
</tr>
<tr>
<td>Mouse gp100 R</td>
<td>TCAGACCTGCTGTCCACTGAGGAGCGGGCT</td>
</tr>
<tr>
<td>mTRP-2 F</td>
<td>ATGGGCCCTTGTTGGGATGGGGCTTC</td>
</tr>
<tr>
<td>mTRP-2 R</td>
<td>CTAGGGCTTCCTCCGTGTATCTCTTG</td>
</tr>
</tbody>
</table>

**Table 5: Primers used to identify SACM tumors**

Primers used to characterize the SCAM tumors for the presence of melanoma markers.

**Tumor challenge**

The k14-SCF mice were treated with 1.5M of MBEH or vehicle for 3 weeks after removing the pelage by application of Nair Church and Dwight Co., Princeton, NJ. A
100μl volume of 5x10^5 B16F10 melanoma cells in PBS was injected subcutaneously in the right flank of MBEH treated and control mice. Tumor volume was recorded daily from day 10 onwards using calipers. Data were pooled from two independent experiments for a total of 8 vehicle treated and 10 MBEH treated animals. Statistical significance of differences among treatment groups was evaluated using Student’s t-test.

**Processing of tumor samples**

Tumors biopsies from SCAM mice or B16 challenged k14-SCF mice were frozen in OCT and stored at -80°C. SCAM mouse tumors were also digested in part in a cocktail of thermolysin, DNase, collagenase and trypsin overnight to obtain single cell suspension to be analyzed for the presence of melanoma markers through FACS and immunohistochemistry.

**Real time PCR**

Cytokine profiling was performed in remaining subcutaneous tumor tissue from B16 challenged mice. Sample sizes were 3 per group for remaining tumor tissue obtained from vehicle and 1.5M MBEH treated animals. RNA was isolated from tissue sections using TRIzol reagent and further purified using an RNeasy mini kit. Two μg of RNA was reverse-transcribed with superscript III and 10% of resultant cDNA was included in the real time PCR reaction. Real time PCR was performed using Quantitect SYBR green master mix and samples were run in triplicate and amplified at 95°C for 10 minutes, [95°C for 15 seconds, 55°C for 30S, 72°C for 30S for 50 cycles]. Standard mouse primer sets and probes for IFNγ, IL-2, IL12b, IL17a, TNFα, IL-10, IL-4, GAPDH and CD3 were
purchased from Qiagen (location). Relative fold change in gene expression was calculated using the \( \Delta\Delta \text{Ct} \) method after normalizing with vehicle treatment (196). Experiments were repeated twice with tumor samples. Representative data from a single experiment are shown.

**Immunohistology**

SCAM tumor cells, B16 melanoma cells, and MOVAS cells (mouse smooth muscle cells) (American Type Culture Collection (ATCC), VA) were plated in gelatin coated chamber slides (Fisher Scientific, city, PA) to adhere overnight. Cells were fixed in cold acetone for 10 minutes (Fisher). Fixed cells were stained with monoclonal antibody Ta99 to TRP-1 (Covance, Dedham, MA), and monoclonal antibody HMB45 to gp100 (Dako, Carpinteria, CA) for 1 hour. Individual alkaline phosphatase labeled or peroxidase labeled isotype specific goat anti mouse antibodies were used as secondary sera. The red color for TRP-1 and blue color to HMB45 was developed using aminoethyl carbazole (Sigma) or fast blue BB (Sigma) as substrates. Images were captured by an Olympus BX41 bright field microscope (Olympus, Center Valley, PA).

Tissues from 3 remaining tumor/group were engaged in the evaluation of infiltrating CD8\(^+\)T cells. Tumor sections were stained with biotinylated 53-6.7 rat antibody to CD8a (Biolegend, San Diego, CA). Anti-rat alexa flour 568 (Invitrogen, Carlsbad, CA) was used as a secondary antibody. Images were captured by an Olympus fluorescence microscope (Olympus, Center Valley, PA).
Results

Genotyping of F2 generation for loss of Ink4a/Arf gene

Homozygous loss of the Ink4a/Arf gene in the F2 generation after backcross with Tyr H RAS Ink4a/Arf-/- mice was assessed by PCR from the DNA obtained from the tail snips of selected male and female mice. Since the neomycin cassette was used as a selection marker for knockout of the Ink4a/Arf gene, the presence of neomycin and the absence of the Ink4a/Arf gene both confirmed the loss of Ink4a/Arf. In addition, the presence of human RAS was observed only in the male offspring confirming the integration of RAS transgene in the Y chromosome as shown in Fig 20.
Fig. 20 Genotyping of SCAM mice

Genotyping of SCAM males and females for the loss of Ink4a/ARF and presence of human RAS. N represents the selection marker neomycin, I-2 represent Ink4a/ARF gene amplified with the primer designed for exon-2, I-3 represent Ink4a/ARF gene amplified with the primer designed for exon-3. R represent human RAS gene and G represent GAPDH, which serves as loading control. The bands of correct size are noted by rectangular boxes. Representative experiment of screening performed on more than 20 mice.
Tumors in SCAM model

The SCAM male mice did generate tumors around 17-18 weeks, however they were not pigmented suggesting that they might be non-melanoma tumors. A representative male mouse displaying a cutaneous tumor is shown in Fig.21.

Validation of SCAM model

The tumor cells dissected from the male SCAM mice were digested in the enzyme cocktail to obtain single cell suspensions. Isolated tumor cells were analyzed for the expression of melanocyte markers both at RNA and protein level.

a) FACS analysis of melanoma markers in SCAM tumor cells

Expression of melanoma associated antigens (MAA) was assessed by staining with anti-TRP-1, anti-tyrosinase, anti-TRP-2, anti-gp100 and anti-MART-1 antibodies and subsequent FACS analysis. Tumor cells from SCAM mice did not express the melanoma associated antigens seen in mouse melanocytes. These results indicate that SCAM tumor are not of melanocyte origin (Fig.22). The experiment was repeated in tumors isolated from the back, ear, eye and tail of 4 mice and similar results were obtained.
Fig. 21 Non pigmented tumor in SCAM mouse

Cutaneous Tumor in SCAM mouse indicated by an arrow. The SCAM mice are on k14-SCF background thus showing pigmented skin, but upon excision the tumor was not pigmented.
Fig. 22 FACS analysis of SCAM tumor cells for presence of melanoma markers

FACS analysis of SCAM tumor cells stained with cocktail of antibodies against TRP-1, tyrosinase, HMB45, gp100 and MART-1. Mouse melanocyte cultures were used as positive control. Grey peak represent isotype control, while the red peak represent antibody staining.
b) Melanoma markers analysis by immunohistochemistry

SCAM tumor cells plated in chamber slides and stained for the presence of gp100 (HMB45; blue) and TRP-1(red). While B16 melanoma cells express both gp100 and TRP-1 seen as purple stain, SCAM tumor cells lacked the expression of either gp100 or TRP-1 (Fig.23), further confirming that the tumors are not melanomas.

c) PCR analysis of melanoma markers at RNA level

RNA isolated from SCAM tumor and mouse melanocytes was reverse transcribed and amplified using specific mouse primers to full-length TRP-2 and gp100. No amplification was observed in the cDNA derived from the RNA of SCAM tumor cells, while the targets were successfully amplified from the positive controls (Fig.24).
Fig. 23 Immunohistochemistry of SCAM tumor cell

SCAM tumor cells stained for gp100 (blue) and TRP-1 (red) expression. B16F10 mouse melanoma cells served as a positive control and MOVAS cells (mouse smooth muscle cells) served as a negative control.
Fig. 24 Amplification of melanoma markers in SCAM tumor cells

Primers were designed to amplify full length TRP-2 and gp100 from cDNA obtained from SCAM tumor cells and mouse melanocytes. Mouse melanocytes served as positive controls. Expected product sizes are 1611 and 1878 for TRP-2 and gp100 respectively as boxed in the Fig.24.
d) Identification of melanoma markers by Western blotting

Proteins isolated from two SCAM tumors (tail and back), B16 melanoma cells and wild type mouse kidney cells were separated in SDS-PAGE gel and probed with antibodies to TRP-1 (Red). SCAM tumor cells did not demonstrate expression of TRP-1, while the mouse melanoma cells did express TRP-1 (Fig.25)
Fig. 25 Identification of melanoma markers in SCAM tumor cells by Western blotting

Proteins isolated from SCAM tumor cells, B16F10 mouse melanoma cells (positive control) and wild type mouse kidney cells (negative control) were separated and stained
for TRP-1 (red). Expected band size for TRP-1 is 70KD. TRP-1 staining in the positive control indicated by a red box. Mouse kidney cells show non-specific band from staining with an irrelevant antibody to S100 in the same blot in blue. Fig 25a represent the whole blot and 25b represent lanes of interest.
**Generation of black (non-agouti) SCAM mice**

The Ink4a/Arf -/- Y Tyr RAS transgenic mice were originally generated in albino mice on an agouti background. Since the F1 offsprings from Ink4a/Arf -/- Y Tyr Ras mice and k14-SCF Tg mice were backcrossed with Ink4a/Arf -/- Y Tyr RAS Tg mice, the offspring were all agouti in trait. The agouti signaling protein acts on melanocortin receptors thereby reducing tyrosinase activity and melanogenesis(210). Since the tyrosinase promoter in SCAM mice should drive oncogenic RAS, we hypothesized that the agouti signaling protein can act as a negative switch for the expression of oncogenic RAS, leading to formation of non-melanoma tumors. Therefore, the agouti trait was eliminated by selecting only the black offspring among F2 mice. Black mice with skin pigmentation along with loss of Ink4a/ARF were selected for future breeding. Male mice were maintained until cutaneous tumors developed. Resulting tumors were non-pigmented and did not express any melanoma markers suggesting that presence of agouti was not responsible for the lack of expression of RAS from the tyrosinase promoter (Fig.26).
Fig. 26 Generation of black SCAM mice

Non-agouti pigmented male mouse displaying a non-pigmented tumor growing through the skin represented by a black arrow.
Characterization of Ink4a/ARF -/- Y Tyr Ras transgenic mice

Cutaneous tumors from parental male Ink4a/Arf -/- Y Tyr HRAS transgenic mice were characterized to confirm melanoma development in parental males.

a) Melanoma markers analysis by immunohistochemistry

Immunohistochemical analysis of cultured tumor cells from Ink4a/Arf male mice using antibody HMB45 to gp100 and Ta99 antibody to TRP-1 did not show any staining suggesting that the tumor cells are non melanomas (Fig.27a). Similar results were obtained upon staining of cryosections obtained from tumor tissues from Y Tyr H RAS Ink4a/Arf -/- male mice with anti-TRP-1 antibody. Staining of a normal eye of SCAM mice served as positive control. (Fig.27b).
**Fig. 27 Immunohistochemical analysis of Ink4a/ARF tumor**

a) Tumor cells obtained from Ink4a/Arf-/- male mice were stained for the presence of gp100 (blue) and TRP-1 (red). B16F10 melanoma cells served as a positive control and MOVAS cells served as a negative control.

b) Tumor sections from male albino Ink4a/Arf-/- Tyr Ras mice stained to detect TRP-1 protein using immunohistochemistry. Normal eye tissue of a SCAM mouse is used as positive control. Hematoxylin was used as a counter stain.
b) Identification of melanoma markers by Western blotting.

Tumor cells obtained from male Ink4a/Arf-/- Y Tyr Ras transgenic mice were separated in an SDS-polyacrylamide gel and stained for the presence of TRP-1 protein (red). Similar to the immunohistochemical analysis, the tumor cells lacked the presence of TRP-1 staining, however the B16F10 melanoma cells showed the expression of TRP-1 (red) with a correct molecular weight of 70KD (Fig.28).
Fig 28 Western blot of Ink4a/Arf-/- male mice tumor

Proteins from B16F10 melanoma cells (positive control), kidney cells from a wild type mouse (negative control) and Y Tyr HRAS Ink4a/Arf-/- male tumor cells stained for the presence of TRP-1 (70KD-red). Boxed band represent proteins of correct size. This
western blot was performed as part of the experiment shown in Fig.23 and the same control lanes were shared between both experiments. As mentioned previously, blue bands represent non-specific staining in control kidney cell and Y Tyr HRAS Ink4a/Arf-/- male mouse tumor cells in blue.
c) Presence of RAS gene under tyrosinase promoter

To identify whether RAS is expressed under the tyrosinase promoter in Ink4a/Arf-/- Y Tyr RAS mice, attempts were made to amplify the transgenic Tyr-RAS construct. The upstream primer was designed for tyrosinase promoter as supposedly included in the original construct, combined with downstream primers to specifically amplify human Harvey RAS and not endogenous mouse RAS. Lack of any amplification using the Tyr-Ras primer set suggested that tyrosinase promoter may not drive RAS expression. However, human Harvey RAS was successfully amplified from male Y Tyr H RAS Ink4a/ARF-/- Tg mice and not in female mice, supporting that human HRAS DNA is integrated into the Y chromosome (Fig.29). Taken together with the absence of melanoma associated markers from resulting tumors, it is likely that the parental Ink4a/Arf-/- Ras Tg model does not carry the parental genopype and phenotype and is therefore not a useful melanoma model.
Fig. 29 Identification of RAS upstream of tyrosinase promoter in Ink4a/Arf mice

A) Primers designed to amplify the tyrosinase promoter reading in to the human HRAS gene in male mice. DNA from Ink4a/Arf/- mice is shown for the absence of RAS and the Ink4a/Arf genes, in presence of the neomycin insert. TR1, TR2 and TR3 represent different forward primers for the mouse tyrosinase promoter combined with reverse primers for human RAS gene. T represents amplification of the tyrosinase promoter. R represents the human HRAS gene. G stands for GAPDH. Boxed bands represents products of the correct, expected size bands, unboxed bands are non-specific bands.
**Determination of anti tumor immune responses mediated by MBEH**

Anti-tumor immune responses mediated by MBEH were measured by challenging MBEH pre-treated or vehicle treated k14-SCF mice with B16 melanoma cells and comparing tumor progression. In addition, immune infiltration of the remaining tumor tissues was studied.

**a) Cytotoxicity of MBEH to mouse melanoma cells and melanocytes**

To identify whether MBEH mediates direct cytotoxicity towards B16F10 mouse melanoma cells, the viability of cultured B16 melanoma cells and melanocytes (derived from k14-SCF mice) in response to 250, 500 and 900 µM concentration of MBEH for 24 hours was determined in MTT assays. In Fig.30 data are pooled from two independently performed experiments. Vehicle treatment served as a control for both cell types. The viability of mouse melanocytes was reduced by 30%, 48% and 59% upon 250, 500 and 900 µM of MBEH treatment respectively. However, no significant reductions was observed in the viability of B16F10 melanoma cells even at the highest concentration of MBEH. This confirms that mouse melanocytes were sensitive to bleaching agents MBEH as previously shown for human melanocytes (chapter 3), whereas melanoma cells are highly resistant to the same chemicals.
Fig. 30 Cytotoxicity of bleaching agent MBEH towards B16 melanoma cells and mouse melanocytes

Sensitivity of B16F10 melanoma cells and k14-SCf derived melanocytes upon treatment with MBEH. Data from 2 independent experiments were combined with triplicate values for each experiment. Mean and SE were calculated and statistical significance was evaluated by Student’s t-test by comparing the viability of cells upon vehicle and MBEH treatment. ‘***’ indicates p<0.0001.
b) Tumor challenge of k14-SCF mice with B-16 melanoma cells

Tumor volume was measured in MBEH or vehicle pretreated k14-SCF mice upon challenging with B16F10 melanoma cells. Data from two independent experiments were combined as shown in Fig.31. Greater than 70% reduction in tumor growth in MBEH treated mice in comparison with vehicle treatment was observed at days 11, 12, 13 and 14 post challenge (p<0.05). At day 14 an average tumor volume of 511 mm$^3$ was measured for vehicle treated mice whereas MBEH treated mice displayed an average tumor volume of 82 mm$^3$ (p<0.05). Mice were euthanized at day 14.
Fig. 31 Tumor challenge of k14-SCF mice with B16 melanoma cells

Treatment of k14-SCF mice with 1.5M of MBEH or vehicle alone for 3 weeks preceded a tumor challenge with B16F10 melanoma cells subcutaneously in the flank region. The volume of the tumor was measured daily from day-10 onwards. Closed circles represent vehicle treated (control) mice, and open triangles represent the MBEH treated group. Data were pooled from two independent experiments for a total of 8 vehicle treated and 10 MBEH treated animals. Statistical significance of differences among treatment groups was evaluated using Student’s t-test ‘*’ indicates p<0.05
c) **Cytokine profiling of the tumors**

The type of anti-tumor immune response activated by MBEH was identified by quantifying the expression levels of pro and anti-inflammatory cytokines in remaining tumor tissue using qRT-PCR. In parallel with the cytokine environment observed in C57BL/6 mice skin after treatment with MBEH (chapter 3), increased expression levels of TNF-α, IFN-γ, IL-2, IL-12 were observed with a relative increase of 5.1, 3.7, 3.4, 7.1 fold over vehicle treatment, respectively. Interestingly, in addition to a Th1 environment, substantial levels of IL-17 were observed with a 7.6 fold increase over vehicle treatment. Expression levels of IL-4 and IL-10 were also increased 2.8 and 1.9 fold over vehicle treatment respectively, and overall these data suggest a Th1/Th17 cell-mediated and inflammatory immune response corresponding to increased anti-tumor reactivity in tumor from MBEH pre-treated mice (Fig.32a). In Fig.32b, relative fold change over vehicle for pro and anti-inflammatory cytokines was quantified by normalizing to CD3 expression levels. A comparison between Fig. 32a and 32b demonstrates that elevated cytokine expression in tumors from MBEH pretreated mice is due in part to enhanced infiltration by T cells, warranting a further comparison of effector T cell infiltration in either group, as shown in Fig.32.
**Fig32 Cytokine profiling in the tumor environment**

a) Transcript levels of pro and anti-inflammatory cytokines evaluated by qRT-PCR in mouse tumor samples. Levels were expressed as transcript abundance for the cytokine per GAPDH transcript content. The relative fold change in the transcript levels following MBEH treatment is shown upon normalization to vehicle treatment. Mean and standard
deviation were calculated from 3 replicates. Experiments were repeated thrice and data from a representative experiment are shown. b) The relative fold change in the expression level of pro and anti-inflammatory cytokines was quantified over vehicle treatment after normalization with CD3 transcript levels.
**d) Infiltration of cytotoxic T cells in the tumor tissue**

The consequence of a Th1 cytokine environment was further established in the remaining tumor tissue by quantifying the abundance of cytotoxic CD8+T cells in either group. Representative stainings are shown for 3 mice from either group (Fig.33). CD8+T cell infiltration was 4.7 fold increased in remaining tumor tissue from 3 animals compared to tumors resected from 3 vehicle treated animals, which was significant at p<0.01 in Student's t-test. These data suggest that anti-tumor protection provided by MBEH pre-treatment may be attributed at least in part to cytotoxic T cells activity.
Infiltration by CD8\(^+\) T cells was quantified in remaining day 14 tumor tissue from k14-SCF mice pre treated with vehicle or 1.5M MBEH for 3 weeks. Representative microscopic fields are shown above with its quantification in the below panel in Fig. 33.
Discussion

Mice are the most commonly used species to generate melanoma models among vertebrates, as they can be genetically manipulated with relative ease. It should be kept in mind that even when desired phenotype is obtained for a particular mouse model, desired genetic alterations may not be indefinitely stable.

The data presented here demonstrate that our SCAM mice did generate cutaneous tumors, however the tumors lacked expression of melanoma markers, despite the mice being null for the Ink4a/Arf genes, along with the presence of human RAS gene specifically in males. FACS analysis, western blot and immunohistochemical studies of SCAM tumor tissues and cells clearly showed the protein level expression of melanoma markers such as tyrosinase, TRP-1, gp100 or MART-1 were absent. Absence of melanoma markers at the transcript level further validated that the tumor cells were not of melanoma origin.

The agouti signaling protein acts as an inverse agonist for α-MSH thereby affecting the activity of tyrosinase gene (211). Since the SCAM mice were initially generated on an agouti background, it was thus hypothesized that presence of agouti trait reduced the activity of the tyrosinase promoter, thereby inhibiting oncogenic RAS expression. However, lack of pigmented melanoma in the SCAM mice generated on a black background suggested that the tyrosinase promoter may not be driving oncogenic RAS expression. It was shown that activity of oncogenic RAS is critical for the penetrance and maintenance of melanoma in the Ink4a/Arf knockout mouse model (212).
Identification of human Harvey RAS encoding DNA only in the male SCAM mice and not in female animals confirms that the human RAS gene is integrated in the Y chromosome; however, its expression is not likely driven by the tyrosinase promoter.

Taken together with the absence of melanoma associated markers from resulting tumors, it is unlikely that the parental Tyr H-RAS Ink4a/Arf-/- mouse model is an appropriate melanoma model to study treatment efficacy for any given melanoma treatment, including the tumor preventive effects of topical bleaching agent MBEH.

In previous chapters, it was shown that MBEH induces necrotic melanocyte death (chapter 2). Further, in vivo studies, demonstrated that application of MBEH to mouse skin activated a predominantly Th1 mediated immune response (chapter 3). Therefore, it is possible that necrotic death of melanocytes in pretreated skin will trigger an immune response that is powerful enough to keep subsequent tumors in check. This concept was subsequently tested in a tumor challenge model where depigmenting k14-SCF mice upon MBEH treatment was challenged with B16 melanoma cells. The resistance of mouse melanoma cells to MBEH mediated toxicity renders the circumstances unlikely for a direct (biochemical) effect on B16F10 melanoma cells. Significantly, reduced tumor growth upon MBEH pretreatment confirmed an anti-tumor immune response activated by MBEH pre-treatment.

Elevated levels of Th1 and Th17 cytokines in the remaining tumor suggest that the MBEH driven anti-tumor response is mediated, at least in part, by cytotoxic T cells, supported by an inflammatory environment. Finally, marked infiltration of CD8+ T cells
support the concept that significant inhibition of tumor growth upon MBEH pretreatment was primarily due in part to CTL activity.

It has been reported that elimination of melanocytes by MBEH is permanent (184). For melanocytes that are not eliminated by MBEH treatment or due to the immune response that follows, it may be important to drive recall responses in patients where remaining melanocytes malignantly transform at a later time point. This question was addressed by van den Boorn et al in a recent publication where MBEH treatment was combined with adjuvants imiquimod and CpG (153). Anti-tumor reactivity was observed even 165 days after cessation of the treatment, thus supporting lasting effects of MBEH.

In conclusion, in vivo data provide for the concept that topical application of bleaching phenols can serve as a prophylactic agent to support the prevention of tumor growth in familial melanoma patients and patients concerned about tumor recurrences. Such protection is offered in a 2-tiered approach, by eliminating precursor cells of the melanocyte lineage that can otherwise malignantly transform, and by eliciting an immune response to melanoma associated antigens. Such response is required to support the removal of distant melanocytes from sites not exposed to MBEH, as well as to otherwise insensitive melanoma cells that share expression of melanocyte differentiation antigens and may be targeted by an effective immune response to the same. In particular, gp100 and MART-1 and tyrosinase are among the most immunogenic molecules expressed by melanoma cells (115, 213-214). Taken together, the data offer pre-clinical support for the
establishment of topical depigmentation as a potent prophylactic treatment against melanoma.
CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Melanoma is the most aggressive skin tumor. The most effective way to reduce the morbidity and mortality associated with cutaneous melanoma is by identifying the tumor at an early stage when surgical removal of the tumor is still a curative option (49). Although the design of therapeutic drugs to target melanoma is an active field of research, avoiding excessive sunlight remains the only preventive approach for melanoma, in particular for people with a genetic predisposition to tumor development (48).

Inducing a vitiligo-like condition can serve as a preventive treatment for familial melanoma since a striking dichotomy exists between vitiligo and melanoma. T cells recognizing melanosomal antigens were reported both in vitiligo and melanoma conditions, thus suggesting an immune mediated response in the disease pathogenesis (145). An approach derived from this observation include the cloning of high affinity T cell receptors from T cells infiltrating vitiligo skin which may be introduced into lymphocytes from melanoma patients to redirect the anti-tumor response (150). This approach is not yet in clinical stage and is experimentally challenging.

In vitiligo patients, depigmentation of the skin progresses with a pace of, on average, 1% of the skin surface per year (146). Depigmentation is also observed in patients with malignant melanoma that develop a significant adaptive immune response to the tumor.
(215). Although depigmentation is generally not desirable for vitiligo patients, once the disease has advanced beyond approximately 50%, patients can opt for chemical depigmentation to achieve an even skin tone (216). Meanwhile, such depigmentation after vaccine therapy is considered as a positive prognostic factor in case of melanoma patients (217).

Bleaching phenolic compounds 4-TBP and MBEH were reported to induce vitiligo-like depigmentation for patients working in rubber, tannery industries (155, 218). Therefore, we exploited whether these bleaching agents could be used in designing preventive therapy for melanoma.

In order to design a preventive strategy, the bleaching agents should specifically remove melanocytes and well as activate an immune response to target melanocytes at farther sites. Since 4-TBP was already known to induce apoptotic destruction of melanocytes, we wanted to understand whether MBEH is toxic to melanocytes. The data presented here demonstrate that MBEH, similar to, 4-TBP, induces specific melanocyte death when compared to keratinocytes. However, the cytotoxic effects were similar in fibroblasts and melanocytes. These data support the notion that topical application will selectively eliminate melanocytes among epidermal cells, at the same time suggesting that systemic application of either agent is associated with toxicity towards cells other than melanocytes. Interestingly, the mode of melanocyte death mediated by MBEH was identified as necrotic and not apoptotic death as induced by 4-TBP. In addition, 4-TBP and MBEH had a differential response in the pigmentary system, where presence of melanin protected against the toxicity mediated by MBEH and not 4-TBP. MBEH and
not 4-TBP was also shown to upregulate the expression levels of melanogenic enzymes. This suggests that a balance exists between the protective effects mediated by melanin and toxic effects of generated orthoquinones and melanocyte death occurs which ever predominates. This protective effect of melanin upon MBEH mediated cytotoxicity could however be problematic to vitiligo patients as people with a darker skin tone would have relatively less effect upon MBEH mediated depigmentation, however could be beneficial for melanoma patients as people with lighter skin tone offer presents with a greater risk of melanoma. Finally, MBEH but not 4-TBP generates ROS in melanocytes. As it was recently shown that expression of p16Ink4a is required for metabolizing ROS, it is clear that cells lacking expression of this oncogene will be increasingly sensitive to MBEH treatment (219).

Given the different modes of cell death induced by either agent, it is reasonable to assume that the extent of an immune response that follows exposure to 4-TBP or MBEH will differ, (220). Indeed, it was shown that only MBEH and not 4-TBP does activate a potent immune response by causing emigration of Langerhans cells from the human epidermis and infiltration of T cells and macrophages in mice skin supported by an increased Th1 mediated cytokine environment upon topical treatment.

The bleaching phenols like 4-hydroxyanisole and 4s-CAP have been used in the melanoma treatment. However, much progress could not be made by using phenolic bleaching agents as a single agent to treat melanoma because melanoma cells are relatively resistant to these agents. The data presented in chapter 3 shows clearly that
human melanoma cells such as 888, 624.38, A375 and M14 are significantly resistant to both 4-TBP and MBEH in comparison with melanocytes. In contrary, a recent work by van den Broon demonstrates that MBEH sensitizes melanoma cell lines such as mel AKR, mel 136.2 (221). This again emphasizes the necessity of using these chemicals in preventive approach as differential sensitivity is being demonstrated by melanoma cells. Differential sensitivity among normal and tumor cells may be defined by multi-drug resistance transporters that pump the chemicals back out of malignant cells, and phenolic agents may never reach the melanosomal compartment to react with melanosomal antigens (222). Alternatively, a different composition of the melanosomal compartment with progressive loss of melanosomal enzymes may determine their relative insensitivity (223-224). Studies to unravel intracellular trafficking are important to help lift the veil surrounding this topic, and potentially expand the use of phenolic bleaching agents to a therapeutic setting in the future. This is one of several items to be addressed in order to further optimize the promising anti-tumor treatment presented here. Further items are addressed in the paragraphs below.

The process of cellular uptake and whether MBEH and/or 4-TBP are trafficked to melanosomes or elsewhere within the cells can be understood by labeling the bleaching compounds and following the intracellular trafficking in melanoma and melanocytes. The observation that 4-TBP and MBEH binds tyrosinase suggests that at least part of the compounds are trafficked through the melanosome (116, 118).
The trafficking of bleaching compounds into the melanosomes of the melanoma cells can be improved using a “Trojan Horse-like” concept, where phenolic compounds can be packed in microbeads such as chitosan. Chitosan is the alkaline deacetylated product of chitin, which is derived from the exoskeleton of crustaceans (225). Chitosan is widely used in the biological drug delivery system. Melanocytes are capable of phagocytosis using melanosomes which would perform the function of lysosomes to complete endosomal fusion (226). This phagocytic potential of melanocytes may be exploited to deliver the bleaching compounds into the melanoma cells. However, it is also possible that the ultimate destination of bleaching compounds lies outside the melanosome, in which case charged nanoparticles could instead be used to target these compounds into the melanoma cells(138).

In order for a lasting bleaching effect to protect against future tumor occurrences, it is important that the bleaching compounds also eradicate undifferentiated melanocyte stem cells. Occasional repigmentation in vitiligo patients upon MBEH treatment suggests that melanocyte stem cells are insensitive to MBEH. In recent studies performed by the Johnson lab, compounds that are capable of preventing melanocyte differentiation in zebrafish have been identified (227). A 1280-compound screening identified novel agents, affecting melanocyte differentiation and viability. The identification of 8-DPAT suggests that treatment with this agent is capable of eliminating cells determined to become melanocytes but not yet sensitive to compounds affecting melanogenesis. Also, compounds papaverine and isoliquiritigenin affect c-kit induced migration of melanocyte
stem cells. These compounds can be tested either solely or synergistically with MBEH in mice and in human explant cultures to identify their impact on melanocyte physiology.

The depigmenting effects of MBEH are well established in vitiligo patients, however, it was not known whether similar depigmentation effects could be achieved targeting melanocytes in control individuals, since it is generally believed that vitiligo melanocytes are specifically vulnerable and increasingly sensitive to external stress compared to healthy melanocytes. The depigmentation studies performed in wild type C57BL/6 mice and k14-SCF mice clearly showed that MBEH can induce significant depigmentation in healthy melanocytes and not just in vitiligo patients. Preliminary studies conducted in the lab to identify the relative sensitivity of normal and vitiligo melanocytes upon treatment with MBEH or 4-TBP indeed showed no intrinsic differences in sensitivity (data not shown). However, it is possible that true differences can be observed only under limiting culture conditions or that further ex vivo or in vivo studies are most suited to understand the intrinsic differences in their sensitivity levels. This can be done using vitiligo mouse models, that develop spontaneous vitiligo due to the expression of a transgenic T cell receptor reactive with mouse tyrosinase (228).

It will be of interest to explore whether melanocytic nevi cells are sensitive to MBEH application. Patients predisposed to melanoma development have a tendency to express greater than 50 nevi at any time (48). Since these nevi carry the primary burden of transformation risk, depigmentation induced nevus removal may be a direct method to remove benign precursor lesions while inducing a more prominent immune response. As
we have shown that melanin offers a level of protection towards toxicity mediated by MBEH (chapter 2), the immune effects may require further boosting using adjuvant therapy such as CpG and imiquimod that was shown to offer therapeutic anti-tumor reactivity for extended periods of time (153). Since topical application of imiquimod have shown to target the Lentigo melanocytes, combination of MBEH and imiquimod might activate a suitable environment as MBEH targets the melanocytes and imiquimod boost the TLR 9 mediated immune response(229)

Taken together, the data offer pre-clinical support for the establishment of topical depigmentation as a prophylactic treatment against melanoma. MBEH may therefore be of direct use for patients with FAMMM (familial atypical multiple mole melanoma syndrome). To test, its prophylactic potential, further evaluation can be performed in pigmented mouse melanoma model. Failure of the mouse tyrosinase promoter to drive the human RAS expression might have resulted in the generation of unpigmented cutaneous tumors. A new-pigmented melanoma mouse model may be generated by crossing mice with oncogenic human N RAS driven by mouse tyrosinase promoter on an Ink4a/Arf null background to k14-SCF tg mice. This model include will better represent human melanomas, as individuals with melanoma frequently have N RAS mutations instead of H RAS. In addition, spontaneous melanomas generated in Tyr N RAS Ink4a/Arf-/- mice with pigmented skin are expectedly metastatic in nature.

In conclusion, our data support that the greatest potential of bleaching phenols in anti-tumor treatment revolves around prophylactic applications.
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VITA

The author, Vidhya Hariharan was born in Chennai, India on November 4, 1980 to AnanthaNarayanan Muthuswamy and Saraswathy Sankar Iyer. Vidhya received her Bachelors and Masters in Biochemistry from the University of Madras. During her undergraduate research work, she focused on studying red blood cells (RBC) and identified premature RBC senescence in diabetic patients. During her undergraduate and graduate studies, she performed summer internships in prestigious hospitals and Universities in Chennai and was selected to enroll in the Honors program in Biotechnology offered by the University of Madras.

In August 2006, Vidhya entered the doctoral program of the Department of Cell Biology, Neurobiology, and Anatomy in the Division of Molecular and Cellular Biochemistry at Loyola University Medical Center. She joined the lab of Dr. Caroline Le Poole in June of 2007 where she began her doctoral work on generating a chemo preventive treatment modality for people with a predisposition to melanoma, using skin-lightening agents. In 2010, Vidhya was awarded a competitive Arthur J. Schmitt dissertation fellowship to successfully complete her graduate work. Her research work has been published in the Journal of Investigative Dermatology and in Melanoma Research. She has also presented her studies in several national level scientific
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Vidhya is married to Hariharan Subramanian, Ph.D, a Research Associate at Northwestern University and co-founder of NanoCytomics, an Evanston-based medical device start-up. They have a wonderful seven month old son, Akshath Hariharan. After her graduation, Vidhya will be joining Avon Global Research, Suffern, NY as a Research Scientist starting June 2011.
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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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