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## ENHANCED BLEACHING TREATMENT: OPPORTUNITIES FOR IMMUNE-ASSISTED MELANOCYTE SUICIDE IN VITILIGO

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### Abstract

Depigmentation in vitiligo occurs by progressive loss of melanocytes from the basal layer of the skin, and can be psychologically devastating to patients. T cell-mediated autoimmunity explains the progressive nature of this disease. Rather than being confronted with periods of rapid depigmentation and bouts of repigmentation, patients with long-standing, treatment-resistant vitiligo can undergo depigmentation treatment. The objective is to remove residual pigmentation in order to achieve a cosmetically acceptable result- that of skin with a uniform appearance. In the USA, only the use of mono-benzyl ether of hydroquinone (MBEH) is approved for this purpose. However, satisfactory results can take time to appear, and there is a risk of repigmentation. MBEH induces necrotic melanocyte death followed by a cytotoxic T cell response to remaining, distant melanocytes. As cytotoxic T cell responses are instrumental to depigmentation, we propose that combining MBEH with immune adjuvant therapies will accelerate immune-mediated melanocyte destruction to achieve faster, more definitive depigmentation than with MBEH alone. Since Toll-like Receptor (TLR) agonists-imiquimod, CpG, and Heat Shock Protein 70 (HSP 70)-all support powerful Th1 responses, we propose that using MBEH in combination with these agents can achieve superior depigmentation results for vitiligo patients.

### Keywords

vitiligo; monobenzone; bleaching phenols; T cells; imiquimod; CpG; HSP70

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### Conflicts of Interest

Authors of this manuscript have no conflicts of interest to declare.

KCW wrote the manuscript, JME designed the figure and provided edits, VH generated proof of concept and gathered references, CH provided critical clinical insight, RML and ICLP designed the hypothesis, contributed research, and edited the manuscript.

## Introduction

Vitiligo is a pigmentary disorder resulting from a loss of melanocytes (1), which varies in its extent of skin involvement and has an unpredictable course (2). Depigmentation can be devastating, having a negative impact on mental health and quality of life (3). Although vitiligo is a multifactorial disease, autoimmunity is the predominant etiologic factor. This is supported by multiple findings (4), including its common association with other autoimmune diseases (5). Strong support exists for T cell-mediated immunity in vitiligo. Increased CD8<sup>+</sup>/CD4<sup>+</sup> infiltrating T cell ratios are observed within perilesional areas of the skin in vitiligo patients (6), and cytotoxic T cells are capable of recognizing melanocytes, leading to apoptosis (7).

Despite its discovery decades ago and its noteworthy prevalence worldwide, no single treatment works effectively in all patients (3). When repigmentation treatments fail and patients have extensive skin involvement, depigmentation therapy can serve to restore a uniform appearance to the skin (8). Currently, mono-benzyl ether of hydroquinone (MBEH) is the sole USFDA approved agent for this purpose (9) and has been widely used as a topical depigmenting agent for vitiligo patients (8,10). The gradual effects, typically notable after four to twelve months of use, are undesirably slow for patients (11). Furthermore, although depigmentation is known to spread beyond the application site, MBEH treatment may not eliminate all melanocytes beyond the application site and carries a risk of repigmentation (12). Here, we propose to combine two synergistic mechanisms: the acceleration of autoimmunity and melanocyte death initiated by MBEH by amplifying T cell responses through immune adjuvants to induce effective, long-lasting, and universal depigmentation.

## Monobenzene Treatment

MBEH is the active ingredient in benoquin cream and is typically formulated in a concentration of 20%. It is a slow-acting depigmenting agent inducing a type IV delayed hypersensitivity response (13). MBEH induces necrotic cell death in epidermal melanocytes (12,14). Similar to findings in mice (15), areas of human skin exposed to MBEH showed cytotoxic CD8<sup>+</sup> T cell infiltrates, supporting that MBEH induces a cytotoxic T-cell immune response that further contributes to depigmentation (16). MBEH exposure leads to production of reactive oxygen species in pigmented cells and increases the release of CD63<sup>+</sup>, tyrosinase<sup>+</sup>, and MART-1<sup>+</sup> exosomes, which can induce specific immunity (17). TLR agonists cytosine-guanine oligodeoxynucleotides (CpG) and TLR7-agonist imiquimod, and inducible heat shock protein 70 (HSP70i) all effectively stimulate the autoimmune response to melanocytes (18,19). Here, we follow the autoimmune concept of vitiligo pathogenesis to address the opportunity of adjuvant-enhanced bleaching treatment. We propose that the combined use of adjuvants, and MBEH, will accelerate the immune-mediated destruction of melanocytes and restore a lasting uniform appearance to the skin.

## Melanization

Melanocytes differentiate from neural crest-derived stem cells, which migrate to the epidermis, hair follicles, choroid of the eye, iris, leptomeninges of the brain and stria vascularis of the cochlea (20). Within melanosomes, melanocytes produce melanin, the

pigment responsible for skin and hair color (21). Melanosomes can synthesize two types of pigments: eumelanin, a dark brown-to-black insoluble polymer, and pheomelanin, a light red-yellow sulfur-containing soluble polymer (22). Both are synthesized through a series of oxidative steps from the precursor molecule, tyrosine. The first step involves conversion of tyrosine to L-DOPA, catalyzed by the enzyme tyrosinase, which is the rate-limiting step in pigment production (23). L-DOPA is then oxidized to dopaquinone, and after this the pathways diverge (24). In eumelanogenesis, dopaquinone is sequentially converted to leukodopachrome, to dihydroxyindole (DHI), and DHI carboxylic acid (DHICA), which polymerizes to form eumelanin (24). In pheomelanogenesis, however, dopaquinone reacts with cysteine or glutathione to form cysteinyl-dopa or glutathionyl-dopa, polymerizing into pheomelanin (24). The ratio of these two pigments in any given cell is determined by the availability of substrates and enzymes responsible for later steps in eumelanogenesis, including tyrosinase-related protein 2 (TRP2) and gp100 (20,25,26).

Fully melanized melanosomes are transferred to adjacent keratinocytes, where they may be arranged in a supranuclear cap to protect nuclear DNA from ultraviolet irradiation (27). Though both pigments absorb UV, eumelanin is more suited for this. Pheomelanin can predispose cells to UV damage by its tendency to produce reactive oxygen species upon UV exposure (28). Hence, melanomas occur more commonly in pheomelanized individuals (29), although the absolute number of melanocytes in healthy individuals is similar among skin types (20).

### Autoimmune pathogenesis of vitiligo

Autoimmunity drives progressive depigmentation in vitiligo. Melanocyte-reactive CD8<sup>+</sup> T cells may be more abundant in peripheral blood of patients with progressive vitiligo than in healthy individuals (30,31), with significant amounts of T cells recognizing melanocyte differentiation antigens MART-1, tyrosinase, and gp100 (7,30,32). Perilesional skin infiltrating CD8<sup>+</sup> T cells also recognize melanocyte antigens and displayed cytotoxicity towards autologous melanocytes (7,33). Reduced local immunosuppression also plays a role in vitiligo. Tregs are less effectively chemo-attracted to the skin in vitiligo patients (34). Because Tregs will suppress self-reactive T cells that escape negative selection in the thymus (35), their decreased presence in vitiligo skin allows for continued activation of cytotoxic T cells, causing widespread melanocyte destruction (36). Th17 cells have also been implicated in vitiligo pathogenesis. Increased serum IL-17 levels in patients correlated with the body surface area (BSA) involvement of vitiligo (37).

Bleaching treatment is likewise associated with inflammatory cytokine expression. Both 4-tertiary butylphenol (4-TBP) and MBEH increase the expression of transcription factor X-box binding protein 1 (XBP1), which activates the unfolded protein response in melanocytes (38). This results in increased production of IL-6 and IL-8 by melanocytes (38). Increased IL-8 expression was likewise found in skin of patients with active disease (39). Overproduction of pro-inflammatory cytokines and decreased availability of immunosuppressive regulatory T cells, combined with increased abundance of cytotoxic T cells, all contribute to depigmentation.

## Mechanism of action of topical depigmenting agents

Topical bleaching phenols, such as MBEH, 4-TBP, and 4-methoxyphenol (also known as 4-MP, mequinol, 4-hydroxyanisole), were found to cause skin depigmentation and are melanocytotoxic (40–42). Other phenolic melanocytotoxic depigmenting agents include 4-tertiary amyl-phenol, 4-tertiary butylcatechol (4-TBC), and 2,4 di-tertiary butylphenol (DTBP) (43–46). Phenolic compounds have structural similarities to tyrosine and can bind to the enzyme's active site (47). Phenols have maximal depigmenting activity with a non-polar side chain in para-position (48). These phenolic compounds serve as alternate substrates for the enzyme tyrosinase to be enzymatically converted into cytotoxic quinones (49), which subsequently bind to cysteine residues in proteins (17,50,51). For example, 4-hydroxyanisole (since renamed 4-methoxyphenol, 4MP) is selectively incorporated into melanosomes, coming in contact with tyrosinase (40). This results in formation of an orthoquinone, which can bind tyrosinase to generate a new compound (52,53). Covalent binding of the newly generated orthoquinone to tyrosinase may generate a neoantigen (17). This haptenization and neoantigen generation process characterizes all aforementioned topical bleaching phenols. Neoantigens can induce autoimmunity by activating immune reactivity to both the modified and unadulterated autoantigens, resulting in CD8<sup>+</sup> cytotoxic T cell migration to the skin and progressive destruction of drug-exposed and unexposed melanocytes (17). Melanocytes are absent from depigmented lesions exposed to 4-TBP (54), as 4-TBP induces melanocyte apoptosis (42). 4-TBP is preferentially cytotoxic to melanocytes (42). In patients exposed to 4-methoxyphenol, melanocytes were likewise decreased in number, histologically resembling vitiligo, and 4-MP cream achieved complete depigmentation in most patients (8).

MBEH was used in the 1930s to prevent the oxidation of rubber, but workers wearing the MBEH-containing rubber gloves soon noticed depigmentation of skin in contact with the gloves (55). The interaction of MBEH with melanocytes triggers a series of cellular effects (oxidative stress, alteration of cellular proteins and increased release of melanoma and melanocyte antigens) that selectively induce immunity against melanocytes and melanoma cells, sparing other cell types. Treatment was found to activate dendritic cells and recruit melanocyte-reactive cytotoxic T-cells to the skin, leading to local and distant melanocyte destruction, even within areas not initially exposed to the MBEH (17,56). Indeed, manufacturers noticed that prolonged exposure to the gloves led to depigmentation in areas of the body not originally exposed to the gloves (55), supporting ongoing autoimmune reactivity upon MBEH exposure (57). T cell clones reactive with MBEH-exposed melanocytes also react with unexposed melanocytes (17), indicating that T cells cross-react with normal melanosomal antigens. These findings suggest that MBEH exposure lowers the threshold for immune reactivity to melanocytes, leading to CD8<sup>+</sup> T cell-mediated depigmentation at distant sites. An inverse relationship was found between sensitivity of melanocytes to MBEH and pigmentation levels, suggesting reduced efficacy in patients with ethnic skin (14). Other skin-whitening agents include hydroquinone, arbutin, tretinoin, alpha-hydroxy acids, kojic acid, azelaic acid, vitamin C, and flavonols (58). These are less relevant to the current strategy, as these agents primarily target tyrosinase activity and interrupt melanization without toxicity to melanocytes (58–64). Thus, permanent

depigmentation cannot be established, and these agents are unlikely to affect melanocyte immune recognition.

## Immune activation by adjuvants

Immune adjuvants imiquimod, CpG, and Heat Shock Proteins have been used to stimulate desirable anti-tumor immune responses (18,65,66). These agents can amplify existing immune mechanisms that mediate melanocyte destruction. Toll-Like Receptors (TLRs) are transmembrane intracellular receptors that bind pathogens and consist of glycoproteins that function as members of the innate immune system, and offer a primitive means to distinguish self from non-self (67). TLRs are found on various cell types, including antigen presenting cells (68). Different TLR subfamilies bind different ligands (69). This leads to the production of inflammatory cytokines (70), promoting lymphocyte activation, and contributing to melanocyte destruction. TLRs induce host cell apoptosis (71) and enhance adaptive immune responses (72–74).

Imiquimod is an immune response modifier approved for topical treatment of anogenital warts, which is also beneficial for treatment of basal cell carcinomas and actinic keratoses (75,76). Upon topical application, imiquimod treatment can induce local vitiligo-like hypopigmentation (77). When applied to lesions such as warts and actinic keratoses, imiquimod acts as a TLR7 agonist (75,78), inducing production of inflammatory cytokines in macrophages. As these inflammatory cytokines can destroy healthy tissue, imiquimod is applied only to the active lesion to minimize healthy tissue destruction. Activation of proinflammatory cytokines promotes proliferation and maturation of naïve T cells and their differentiation into Th1 lymphocytes (67). Thus, imiquimod indirectly promotes cytotoxic T cell responses. Imiquimod also promotes the maturation of epidermal Langerhans cells, which leads to increased antigen presentation and immune activation (79). Focally, imiquimod induces melanocyte apoptosis (80) by downregulating microphthalmia-associated transcription factor (MITF), a gene regulating melanocyte survival and transcription of melanocytic proteins (81–84). MITF downregulation results in decreased Bcl-2 transcription and a predisposition to apoptosis.

CpG oligonucleotides (CpG-ODNs) are TLR9 agonists (66). TLR9 recognizes unmethylated CpG DNA segments, which was the premise for their use in anti-tumor immunotherapy (66). In mice, CpG oligodeoxynucleotide combined with tumor-specific peptide effectively induced a Th1 tumor immune response (85,86). CpG-ODNs cause potent Th1-type cytokine responses and increased expression of major histocompatibility complex (MHC) molecules and costimulatory molecules on antigen-presenting cells (87).

Heat shock proteins (HSPs) are upregulated in response to stress (88) and serve to chaperone existing peptides and nucleotides to prevent misfolding and apoptosis, when overall protein synthesis is halted (88). When HSPs are released from cells, antigen presenting cells receive this danger signal to promote an inflammatory response (89), in part by increasing the synthesis of Th-1 cytokines (90). Inducible HSP70 can be secreted by live cells (91) and is expressed by melanocytes under stress (88). HSP70 activates the innate immune system, functioning as an endogenous ligand of TLR2 and TLR4 (92,93). Secretion of HSP70i by

stressed melanocytes causes dendritic cell activation to mount autoimmune responses against melanocytes (94). Indeed, vaccination with HSP70i encoding DNA resulted in significant and continued depigmentation in vitiligo prone mice (18).

## Enhanced Bleaching Treatment for Vitiligo

MBEH produces suboptimal skin bleaching results. Given the strong evidence supporting autoimmunity in vitiligo pathogenesis, we propose that limitations to MBEH treatment may be overcome with supplementary immune adjuvant therapy to enhance the depigmentation process. Combination therapy including two bleaching agents was previously tested in guinea pigs, namely, MBEH and all-trans retinoic acid (ATRA) (95). Here, we propose the synergistic use of immune adjuvants in conjunction with MBEH. Th1 cytokines activate cytotoxic processes (96), and Th1 lymphocytes mediate delayed-type hypersensitivity responses (97). Imiquimod, CpG, and HSP70 are adjuvants known to promote Th1 responses and can thus be used as immune adjuvants for enhanced depigmentation therapy. The principal mechanism is illustrated in Fig. 1. In mice, monobenzone therapy, combined with imiquimod and CpG, in “MIC” therapy, induced a potent and lasting immune response against melanoma antigens and eradicated melanoma tumors (19). The combination of MBEH and imiquimod without CpG likewise effectively induced anti-melanocyte immunity, and may constitute a more affordable option to accelerate depigmentation in vitiligo patients. Though such combination therapy was effective in mice, its clinical treatment potential for human skin has yet to be demonstrated (19). In light of the continuum of melanocytes in human skin, whereas mouse melanocytes locate to hair follicles, an equal or better treatment efficacy may be predicted for patients.

Importantly, in employing immune adjuvants for the current application, agents should be applicable topically, to avert systemic immune activation with unnecessary destruction of healthy tissues. Currently, imiquimod is the only adjuvant listed above available in a topical formulation. As adjuvant therapy is meant to enhance existing immune responses, and vitiligo patients have heightened anti-melanocyte immune reactivity, imiquimod-assisted MBEH responses are likely to be selectively amplified in vitiligo patients. HSP70i protein has been applied by gene gun vaccination in mice, and passive transdermal migration of full length protein is unlikely to occur. Finally, CpG is not available in a topical formulation for human use and has mostly been studied when administered as an intravenous or subcutaneous injectable in humans. Thus, MBEH plus imiquimod is currently the preferred option for combination treatment.

Note that the expense of common imiquimod formulations is limiting to patients when not covered by insurance companies, providing immediate incentive for further (pre)clinical testing. Fortified MBEH therapy may inadvertently affect melanocyte viability in extracutaneous sites, which could affect hearing or visual acuity; however, melanocytes in immune-privileged sites are relatively protected from such responses. Indeed, there is minimal evidence for hearing or visual impairment in vitiligo (98–100). By contrast, a significantly advantageous effect of this therapy could include targeting of occult melanomas, as supported by previous studies (15,19).



In conclusion, combining MBEH with imiquimod and/or CpG or HSP70 should be considered for enhanced depigmentation therapy of vitiligo patients. We propose that MBEH will work synergistically with the aforementioned immune adjuvants to achieve faster and longer-lasting depigmentation, to produce cosmetic results far superior to those achieved with currently available therapies.

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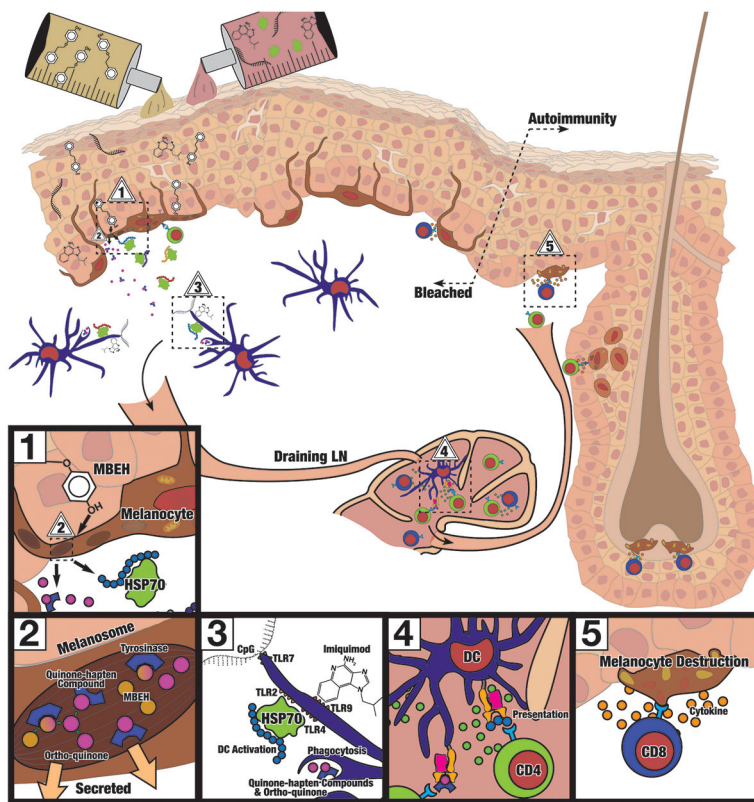
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**Fig. 1. Principle of adjuvant enhanced skin bleaching**

We propose that enhanced depigmentation can be achieved by (1) combined application of bleaching phenols such as MBEH and immune adjuvants such as imiquimod, CpG or HSP70 introduced to the skin. The phenolic agents will (2) traffic to the melanosomes within melanocytes found in the basal layer of the epidermis, where they are converted to toxic ortho-quinones that haptenize the tyrosinase enzyme. The modified antigenic peptides are then released by cells under stress or dying melanocytes to (3) come in contact with dermal dendritic cells in combination with co-applied adjuvants to (4) activate melanocyte-reactive T cells that (5) are recruited to the skin to eliminate remaining pigment cells and establish lasting depigmentation.