Identification of Quinolines that Inhibit Melanogenesis by Altering Tyrosinase Family Trafficking

Li Ni-Komatsu, ChunXiang Tong, Guangming Chen, Nelya Brindzei and Seth J. Orlow

The Ronald O. Perelman Department of Dermatology and the Department of Cell Biology, New York University School of Medicine, New York, NY 10016, USA (L.N.K., C.X.T., N.B., S.J.O.)

PTC Therapeutics, 100 Corporate Court, South Plainfield, NJ 07080, USA (G.M.C.)
Running title: quinoline compounds as pigmentation inhibitors

Corresponding author: Seth J. Orlow, Dermatology Room H-100, NYU School of Medicine, 560 First Avenue, New York, NY 10016, USA
Telephone: 212-263-5245
Fax: 212-263-8752
E-mail: seth.orlow@nyumc.org

Number of text pages: 32
Number of tables: 2
Number of figures: 4
Number of references: 40
Number of words in Abstract: 166
Number of words in Introduction: 542
Number of words in Discussion: 1229

Abbreviations: Tyr, tyrosinase; ER, endoplasmic reticulum; Tyrp1, tyrosinase-related protein 1; Lamp-1: lysosome-associated membrane protein 1; TGN, trans-Golgi network; DMSO, dimethyl sulfoxide; MEL-A: Asian human skin equivalent; MEL-B: African-American human skin equivalent.
Abstract

A series of quinolines including chloroquine and quinine were identified as potent pigmentation inhibitors through screening a compound library in murine melanocytes. Structure-activity relationship analysis indicated that 4-substituted amino groups with a tertiary amine side chain, such as chloroquine, were associated with robust inhibitory activity. In contrast to many previously identified pigmentation inhibitors, these newly identified inhibitors had no effect on either the level or the enzymatic activity of tyrosinase, the rate-limiting enzyme in melanin production. Rather, our results showed that these quinolines inhibited melanogenesis by disrupting the intracellular trafficking of tyrosinase-related proteins and lysosome-associated membrane protein 1 (Lamp-1). In treated melanocytes, tyrosinase and tyrosinase-related protein 1 accumulated in Lamp-1-positive perinuclear organelles instead of melanosomes, thus preventing melanogenesis. The depigmenting abilities of chloroquine and quinine salicylate were assessed in a human skin equivalent model (MelanoDerm). Both compounds were considerably more effective than arbutin, a widely used lightening agent. Our results indicate that quinolines may be useful agents for cosmeceutical skin lightening and treatment of hyperpigmentation disorders.
Introduction

Constitutive skin pigmentation depends primarily on the amount and type of melanin synthesized by melanocytes located in the basal layer of epidermis and its distribution towards the surface of the epidermis (Hearing, 2005; Yamaguchi et al., 2007). Melanin is synthesized within a specialized organelle termed the melanosome (Jimbow et al., 1976; Shen et al., 2001; Hearing, 2005). An essential enzyme in the synthesis of melanin is tyrosinase (Tyr), a melanosomal membrane glycoprotein that catalyzes the initial and rate-limiting steps of melanogenesis (Korner and Pawelek, 1987; Tripathi et al., 1992). After initial synthesis and processing in the endoplasmic reticulum (ER), tyrosinase traffics through the Golgi network and from there to its final destination, the melanosome, where melanin synthesis and deposition takes place (Raposo et al., 2001; Toyofuku et al., 2001). Ultimately, melanins packed in melanosomes are transferred from melanocytes to the adjacent keratinocytes. The distribution pattern of melanosomes and their breakdown in keratinocytes are also processes that play important roles in determining skin pigmentation (Van Den Bossche et al., 2006; Goding, 2007; Yoshida et al., 2007).

The ability to control skin pigmentation may be used to address a variety of medical conditions such as acquired hyperpigmentation. A number of lightening agents, such as hydroquinone, arbutin and kojic acid, are also commonly used as cosmetic additives. However, they are only modestly active and some, like hydroquinone, may be associated with adverse side effects (Abramowitz and Chavin, 1980; Curto et al., 1999). There is a need for more effective and safe lightening agents to be used in cosmetic and medicinal products for the treatment of hyperpigmentation disorders.

Despite remarkable progress in the knowledge of melanocyte biology and the mechanism of melanin synthesis, the regulation of mammalian pigmentation remains incompletely
understood due to its complexity. For example, other types of cells in the skin also contribute to the regulation of melanogenesis, and depigmenting compounds identified by using melanocytes alone in vitro might not prove effective in reducing human skin pigmentation. In recent years, reconstituted human skin equivalents such as MelanoDerm have provided a means to evaluate cosmetic and pharmaceutical agents designed to modulate pigmentation (Yoon et al., 2003; Takeyama et al., 2004; Matsuda et al., 2008). MelanoDerm consists of normal human-derived epidermal keratinocytes and melanocytes which undergo spontaneous melanogenesis in a morphological and growth environment similar to human epidermis. These human skin equivalents may provide a more physiologically relevant system to study the three-dimensional interactions of melanocytes and keratinocytes and to elucidate the regulatory mechanism of pigmentation modulators.

Our laboratory has successfully employed chemical genetic screening to identify novel compounds and their targets involved in the regulation of mammalian pigmentation (Ni-Komatsu and Orlow, 2007; Ni-Komatsu and Orlow, 2008). In the current study, we screened a commercially available library of natural products and marketed drugs for their ability to inhibit pigmentation in cultured murine melanocytes in order to discover alternative treatments for hyperpigmentation disorders. A group of quinolines was identified as potent pigmentation inhibitors. The mechanism by which they inhibit melanogenesis was further characterized and found to involve disruption of tyrosinase family member trafficking. Two compounds, chloroquine and quinine salicylate, were selected to verify their effects on pigmentation in the MelanoDerm model. Our results indicate that compared to arbutin, both quinolines are much more potent melanogenic inhibitors.
Materials and Methods

Materials. Chloroquine, cinchonidine, hydrocinchonine, hydroquinidine, hydroquinidine 4-chlorobenzoate, hydroquinine, hydroquinine hydrobromide hydrate, N-benzylcinchonidinium chloride, mefloquine hydrochloride, primaquine diphosphate, quinacrine hydrochloride, quinidine and quinine were purchased from Sigma-Aldrich (St. Louis, MO 63103). Amodiaquine dihydrochloride, dibucaine hydrochloride and quinine ethyl carbonate were purchased from MicroSource Discovery Inc. (Gaylordsville, CT 06755).

αPep7 antiserum was raised against the C-terminus of mouse Tyr based on reported antigenic peptide sequences (Jimenez et al., 1991). The following antibodies were also used: Mel-5, a monoclonal antibody reactive with mouse Tyrp1 (Signet Laboratories Inc., Dedham, MA 02026); 1D4B, a monoclonal antibody reactive with mouse Lamp-1 (The Developmental Studies Hybridoma Bank, Iowa city, IA 52242); sheep anti-TGN38, a polyclonal antibody reactive with mouse TGN38, a Trans-Golgi network (TGN) marker (Serotec, Oxford, UK).

Compound Library. The library used in this study was The Spectrum Collection (MicroSource Discovery Inc., Gaylordsville, CT 06755). The 2000 compounds in this library include many marketed drugs or natural products, and are supplied as a 10 mM solution in dimethyl sulfoxide (DMSO).

Cell Culture. Murine melan-a cells were cultured as previously reported (Ni-Komatsu and Orlow, 2008).
Screening of the Compound Library in melan-a Cells and Melanin Assay in Cells.

Screening and melanin assay were performed as reported previously (Ni-Komatsu and Orlow, 2008).

Immunofluorescence Microscopy. Cells were cultured on coverslips (Fisher Scientific, Pittsburgh, PA 15219), incubated with or without 2.5 μM chloroquine or mefloquine, 5 μM quindine, quinine, or quinine salicylate for 72 hrs or the indicated time at 37 °C. The cells were fixed with −20 °C methanol for 5 min and stained as described previously (Shen et al., 2001). The slides were analyzed with a confocal microscope (LSM 510, Zeiss, Proctor, MN 55810).

Culture and Treatment of Human Skin Equivalent. MelanoDerm (MatTek Corp., Ashland, MA 01721) is a viable reconstituted three-dimensional human skin equivalent containing normal melanocytes and keratinocytes that are derived from African-American (MEL-B), Asian (MEL-A) or Caucasian (MEL-C) donors. MEL-A and MEL-B tissues were used in the current study, and they were maintained in the NMM-113 medium as recommended by the manufacture.

Chloroquine was dissolved in water to a final concentration of 0.5 mM (equal to 0.26 mg/ml). Quinine salicylate was dissolved in 30% water: 70 % propylene glycol (PEG) to a final concentration of 4.0 mM (equals to 1.85 mg/ml). A 25 μl aliquot of each compound was applied topically to the Asian MelanoDerm tissue (MEL-A) on Days 0, 1, 3, 6, 8, 10 and 13. The MelanoDerm tissues were fed every other day with 5 ml fresh NMM-113. Prior to each application, the tissues were washed with 1 ml PBS to remove any residual test compound. Tissues were fixed on Days 13 and 16 for microscopic analysis and histological evaluation. In addition, duplicate tissues were frozen on Days 13 and 16 for melanin assay. 30% water: 70 %
PEG and water alone were used as negative controls and arbutin (at concentration of 3 mg/ml, approximately 11 mM), was used as positive controls.

Similar experiments were performed on African-American skin equivalents (MEL-B) except that a 25 µl aliquot of each compound was applied topically to MEL-B on Days 0, 1, 3, 6, 8 and 10, and the treatment was lasted for either 10 or 13 days.

**Cell Viability Assay of Human Skin Equivalent.** Cell viability was measured in accordance with the manufacturer’s instructions using the MTT assay kit (MatTek Corp., Ashland, MA 01721). A 10 or 25 µl aliquot of chloroquine (at 0.5 mM) or quinine salicylate (at 4.0 mM) was applied topically to MEL-A on Days 0, 1, 3, 6. 30% water: 70% PEG and water alone were used as negative controls. One MEL-A tissue from each condition was harvested on day 2 and 7, and MTT assay was performed. Similar experiments were performed on MEL-B.

**Melanin Assay of Human Skin Equivalent.** Melanin content in treated human skin equivalent (MEL-A and MEL-B) was determined as previously reported (Rosenthal et al., 1973; Takeyama et al., 2004). The frozen tissues were homogenized in 0.45 ml of 1% SDS, 50 µM EDTA, and 10 mM Tris, pH 6.8. To each homogenate, 20 µl of protease K was added at 5 mg/ml. Digestion proceeded overnight at 45 °C, and an additional 20 µl protease K was added for 4 more hour incubation. Then 50 µl of 500 mM sodium carbonate and 10 µl of 30% H2O2 were added to the homogenates. Samples were maintained at 80 °C for 30 min and cooled to room temperature. The mixture was extracted with 100 µl of chloroform: methanol (2:1). After centrifugation at 10,000 g for 30 min, the optical density of the top phase at 490 nm was measured. Synthetic melanin (Sigma-Aldrich, St. Louis, MO 63103) was subjected to the same
procedure as a control, and a standard curve was constructed so that the melanin content of the unknowns could be determined for each cultured tissue.

**Histological Analysis of Human Skin Equivalent.** Treated African-American and Asian human skin equivalent (MEL-B and MEL-A) samples were fixed with 10% formalin (Sigma-Aldrich, St. Louis, MO 63103) at 4 °C overnight. Samples were observed under phase-contrast microscopy.

To visualize the melanin distribution throughout the tissue, Fontana-Masson silver stain was performed on the paraffin-embedded tissues as previously described (Bancroft and Stevens, 1982; Yoon et al., 2003).
Results

Identification of Quinoline Analogs as Pigmentation Inhibitors by Screening the Spectrum Collection Library in Murine Melanocytes. A commercial library of 2000 drugs and natural products was screened to identify novel compounds that inhibit melanin synthesis in cultured murine melanocytes (melan-a cells). Screening was performed at final compound concentration of 1 μM (0.1 μl of stock) in 24-well plates followed by melanin assay. 0.1 μl DMSO was used as a negative control and the tyrosinase inhibitor phenylthiourea (PTU) at 300 μM was used as a positive control on every plate (Hall and Orlow, 2005). Positive candidates were rescreened in duplicate at final concentrations of 1 and 5 μM, resulting in the initial identification of four quinolines amodiaquine dihydrochloride, primaquine diphosphate, quinacrine hydrochloride and quinine as potential pigmentation inhibitors.

These drugs all belong to a family of quinolines that function as antimalarials (Goodman and Gilman, 1996). Additional quinolines were tested for their effect on pigmentation and seven more compounds, chloroquine, cinchonidine, dibucaine hydrochloride, mefloquine hydrochloride, quinidine, quinine ethyl carbonate and quinine salicylate, were also found to inhibit melanin synthesis to varying extents in melan-a cells. Inhibition of melanin synthesis in a dose-dependent manner in melan-a cells was observed with all eleven tested quinolines (data not shown). Cinchonidine, dibucaine hydrochloride, quinine ethyl carbonate and quinine salicylate did not exhibit cytotoxicity at concentrations below 20 μM; primaquine diphosphate, quinidine and quinine were not cytotoxic at concentration below 10 μM; amodiaquine dihydrochloride, chloroquine and mefloquine hydrochloride did not show any cytotoxicity at concentrations below 5 μM; while quinacrine hydrochloride showed cytotoxicity at concentrations above 2.5 μM (data not shown).
The IC₅₀ values for inhibition of cellular melanin synthesis for the eleven quinolines were determined by culturing melan-a cells with various concentrations of each compound for 72 hours (Table 1). Chloroquine was the most potent pigmentation inhibitor with an IC₅₀ less than 1 μM; amodiaquine dihydrochloride, mefloquine hydrochloride, primaquine diphosphate, quinacrine hydrochloride, quindine, quinine, quinine ethyl carbonate and quinine salicylate were less potent pigmentation inhibitors with IC₅₀ values between 1 and 10 μM; cinchonidine and dibucaine hydrochloride showed the least potency in inhibiting pigmentation with IC₅₀ values above 10 μM (Table 1). By comparison, in our system the IC₅₀ values of two previously recognized pigmentation inhibitors, PTU and hydroquinone, were 29 μM and 36 μM, respectively (Hall and Orlow, 2005). These data suggest that when tested on melanocytes in vitro, all eleven quinolines are more potent pigmentation inhibitors than currently available depigmenting compounds. Although quinacrine hydrochloride was a potent pigmentation inhibitor, it was eliminated from further study due to its high cytotoxicity.

**Structure-activity Relationship Analysis of Quinoline Analogs as Pigmentation Inhibitors.** To gain insight into structure-activity relationships (SAR), additional quinoline analogs were tested for their ability to inhibit melanin synthesis in melan-a cells at final compound concentrations of 1 and 5 μM. The effects on pigmentation in melan-a cells are presented in Table 2.

Our data indicate that 4-substituted amino groups with a tertiary amine side chain provide good inhibitory activity. For example, chloroquine inhibited pigmentation with an IC₅₀ of 0.7 μM. Similar activity was observed for amodiaquine dihydrochloride and quinacrine hydrochloride; both can be considered 4-substituted aminoquinolines where the amino group
bears an additional tertiary amine side chain. Furthermore, compounds having a 4-alkyl group containing a tertiary amine side chain, such as quinine and quinidine derivatives, also exhibited good activity. The chirality of the alpha-carbon of the 4-substituent in quinine and quinidine derivatives does not appear to have any impact on the activity. Finally, substitutions in the phenyl ring of the quinoline core appear to enhance the inhibitory activity. For example, unsubstituted compounds, such as cinchonine and cinchonidine, which are close analogs of quinine and quinidine, showed low (cinchonidine) or no (cinchonine) activity as pigmentation inhibitors.

Quinolines Inhibit Pigmentation via Altering the Intracellular Trafficking of Tyrosinase and Tyrosinase-related Protein 1. As tyrosinase plays a central role in melanogenesis, it is not surprising that many depigmenting compounds act via effects on the expression, processing, maturation, degradation and enzymatic activity of this enzyme (Parvez et al., 2006; Solano et al., 2006; Ni-Komatsu and Orlow, 2008). Therefore, we performed several assays to determine whether these quinoline pigmentation inhibitors affected tyrosinase.

None of the quinolines appeared to alter levels of tyrosinase, Tyrp1 and Tyrp2/Dct proteins as assessed by immunoblotting (data not shown). The effect of these compounds on tyrosinase activity was studied in vitro using melan-a cell lysates. None of the tested quinolines directly inhibited tyrosinase activity (data not shown). No obvious difference in tyrosinase activity of melan-a cell lysates was observed when the cells were pre-incubated with each compound for 72 hrs before harvesting (data not shown). These data suggested that quinolines decreased pigmentation through mechanisms other than inhibiting tyrosinase levels or enzymatic activity.

Many genetic disorders of hypopigmentation are due to alterations in the subcellular distribution of tyrosinase gene family proteins (Chen et al., 2002; Richmond et al., 2005; Wei,
Therefore, compounds that inhibit pigmentation could act through altering the subcellular distribution of these proteins, a phenomenon reported previously by our laboratory (Hall et al., 2003; Ni-Komatsu and Orlow, 2008).

We evaluated the subcellular localizations of tyrosinase and Tyrp1 in melan-a cells treated with quinolines by immunofluorescence (Fig. 1A). As observed previously in these cells (Chen et al., 2002; Toyofuku et al., 2002), in both vehicle-treated, as in untreated cells, tyrosinase and Tyrp1 are found in melanosomes distributed throughout the cytoplasm, as well as in the perinuclear area where the endoplasmic reticulum (ER), Golgi and stage I melanosomes are located. Dramatic differences in the subcellular distribution of tyrosinase and Tyrp1 were observed after treatment of melan-a cells with the newly identified quinoline pigmentation inhibitors. In melan-a cells treated with chloroquine, quinidine, quinine and quinine salicylate, both tyrosinase and Tyrp1 were almost exclusively restricted to the perinuclear region (Fig. 1A). Similar results were observed in melan-a cells treated with each of the other quinoline analogs (data not shown), except mefloquine hydrochloride. In melan-a cells treated with mefloquine hydrochloride, tyrosinase was trapped to the perinuclear region (Fig. 1A), as observed when cells were treated with the other quinolines. However, mefloquine hydrochloride treatment did not appear to alter the subcellular localization of Tyrp1, and the colocalization of Tyr and Tyrp1 was abolished in treated cells (Fig. 1A).

Tyrosinase (Tyr) is a type I membrane glycoprotein with 6 or 7 asparagine residues that undergo N-glycosylation (Korner and Pawelek, 1982; Ujvari et al., 2001). After initial glycosylation and processing in the ER, Tyr traffics through the Golgi network and from there to its final destination, the melanosome (Raposo et al., 2001; Toyofuku et al., 2001). No obvious change in the endoglycosidase H (Endo-H) sensitivity of tyrosinase was observed in melan-a cells treated with these quinolines (data not shown), suggesting that in these cells, tyrosinase has been
processed beyond the ER and the early Golgi compartments. The perinuclear pool of tyrosinase in melan-a cells treated with chloroquine, quinidine, quinine and quinine salicylate showed no obvious colocalization with TGN38, a trans-Golgi network (TGN) marker (Fig. 1B, data not shown for the other quinoline pigmentation inhibitors), indicating that tyrosinase trafficked beyond the TGN in melan-a cells treated with these compounds. Post-Golgi sorting of tyrosinase is thought to involve trafficking through endosomal compartments (Theos et al., 2005).

However, quinoline treatment did not appear to affect the subcellular localizations of either adaptor protein complex 3 (AP3) or mannose 6-phosphate receptor (M6PR) (data not shown), two proteins involved in the transport of melanosomal/lysosomal proteins from TGN to endosomes (Shen et al., 2001; Theos et al., 2005; Ni et al., 2006).

**Induction of Tyrosinase and Lysosome-associated Membrane Protein 1**

**Colocalization by Quinoline Pigmentation Inhibitors.** A common lineage among melanosomes and lysosomes is well accepted (Orlow, 1995; Raposo and Marks, 2002; Dell’Angelica, 2003). Both organelles contain certain proteins in common, and both are affected in several single gene disorders, such as Hermansky-Pudlak Syndrome (HPS) subtype 1 (HPS-1) that alter the subcellular trafficking of lysosome-related organelles (Boissy et al., 1998; Richmond et al., 2005). Our laboratory has reported previously that certain tricyclic compounds such as tricyclic antidepressants and the steroidal compound U18666A inhibit pigmentation by altering the subcellular distribution of both tyrosinase gene family proteins and lysosome-associated membrane protein 1 (Lamp-1) (Hall et al., 2003; Ni-Komatsu and Orlow, 2008). Therefore, the subcellular localization of Lamp-1 in melan-a cells treated with quinoline analogs was also investigated by immunofluorescence.

In untreated melan-a cells, Lamp-1 was localized mainly in cytoplasmic granules with some in the perinuclear area (Fig. 2). No obvious colocalization of Lamp-1 with tyrosinase was
observed in untreated cells, consistent with the previous report that tyrosinase is not detected in
late endosomes/lysosomes in these cells (Hall et al., 2003). By contrast, in the melan-a cells
treated with chloroquine, quinidine, quinine or quinine salicylate, Lamp-1 was localized to the
perinuclear region, similar to that observed for the tyrosinase gene family proteins (Fig. 1). In
addition, a significant colocalization of tyrosinase and Lamp-1 was also observed in the cells
treated with these compounds for 72 hrs (Fig. 2). Similar to its lack of effect on the subcellular
localization of Tyrp1, mefloquine hydrochloride treatment did not appear to have any obvious
effects on Lamp-1 subcellular localization (Fig. 2).

Our cell-based assay data suggested that with the exception of mefloquine hydrochloride,
quinoines tested in this study inhibited melanogenesis by altering the trafficking of tyrosinase
and Tyrp1 in melan-a cells, causing them to be trapped in a Lamp-1-positive perinuclear
compartment. Even though no obvious effects on the intracellular trafficking of Tyrp1 and
Lamp-1 were observed, mefloquine hydrochloride treatment did alter the trafficking of tyrosinase
in treated cells. The correct regulation of intracellular trafficking of tyrosinase and related
proteins plays an important role in melanogenesis, as tyrosinase and Tyrp1 cannot synthesize
melanin if they do not traffic to melanosomes.

**Chloroquine and Quinine Salicylate Inhibit Pigmentation in a Three-dimensional
Asian Human Skin Equivalent Model.** Human skin equivalent models have been used
increasingly to evaluate pigmentation modulators identified through *in vitro* assays for their
abilities to affect pigmentation in a system more closely representative of human skin (Yoon et
al., 2003; Takeyama et al., 2004; Matsuda et al., 2008). We chose two quinoines, chloroquine
and quinine salicylate, for testing on the commercially available human skin equivalent,
MelanoDerm. Their effects on melanogenesis were compared to both vehicle-treated controls
and MelanoDerm treated with arbutin, an agent widely used in cosmeceutical products for the
treatment of hyperpigmentary disorders. Both chloroquine and quinine salicylate were potent pigmentation inhibitors identified in our cell-based screening, with chloroquine standing as the most effective among all the tested quinoline compounds. Second, both chloroquine and quinine salicylate have a long history of human oral usage in the treatment of malaria (Goodman and Gilman, 1996). Finally, neither compound exhibited substantial cytotoxicity to melanocytes.

MelanoDerm is a living, reconstituted, highly differentiated, three-dimensional human epidermal-like tissue containing melanocytes and keratinocytes that are derived from African-American, Asian or Caucasian donors (termed MEL-B, MEL-A and MEL-C, respectively). Both MEL-B and MEL-A tissues were used in the current study. In this model, substantial accumulation of newly synthesized melanin occurs over a two to three week period.

There was a considerable decrease in relative melanin accumulation in Asian human skin equivalents (MEL-A) after topical treatment with either 0.5 mM chloroquine or 4.0 mM quinine salicylate (Fig. 3A). A 40-50% decreased melanin content was observed in MEL-A treated with 0.5 mM chloroquine for both 13 and 16 days. Quinine salicylate was less effective at day 13, but by day 16, a 30-40% lesser melanin content was observed in MEL-A treated with 4.0 mM quinine salicylate. By comparison, the widely used melanogenic inhibitor arbutin at a concentration of 3 mg/ml (approximately 11 mM), did not appear to have an obvious effect on the melanin content of MEL-A after either 13 or 16 days treatment (data not shown). The decreased melanin content in MEL-A treated with either chloroquine or quinine salicylate was not likely due to cytotoxicity. The cell viability of either 0.5 mM chloroquine- or 4.0 mM quinine salicylate-treated MEL-A was examined by cytotoxicity testing performed according to the manufacturer’s instructions, and no detectable cytotoxicity was observed with either compound treatment (data not shown). Neither compound at the indicated concentration appeared to alter cell proliferation or morphology (data not shown).
The effects of chloroquine and quinine salicylate on melanin synthesis in MEL-A were evaluated by light microscopy. A visible difference in pigmentation was observed 13 days after chloroquine treatment, when compared to either the vehicle-treated control or MEL-A treated with quinine salicylate or arbutin (Fig. 3B). In MEL-A treated with chloroquine or quinine salicylate, the degree of pigmentation was strikingly decreased by day 16 when compared to controls (Fig. 3B). This is consistent with the melanin assay data, suggesting that chloroquine is a more effective whitening agent than quinine salicylate. However, compared to arbutin, either quinoline is a more potent melanogenic inhibitor.

The transfer of melanin from melanocytes to keratinocytes and their distribution in keratinocytes are processes that play important roles in the regulation of skin color (Van Den Bossche et al., 2006; Goding, 2007; Yoshida et al., 2007). The distribution of melanin in the treated MEL-A was assessed by image analysis of histological sections stained with the Fontana-Masson method that highlights melanin (Bancroft and Stevens, 1982; Yoon et al., 2003) (Fig. 3C). At both day 13 and 16, chloroquine treatment resulted in a significant decrease in the relative melanin content of MEL-A. While no noticeable difference in melanin content was observed in MEL-A treated with quinine salicylate for 13 days, an obvious decrease was observed with 16 days of quinine salicylate treatment. Quantitative analysis was performed on 8-10 Fontana-Masson stained histological sections of either vehicle-treated or compound-treated Melanoderm. The results indicated a 47.1% (P< 0.001, day 13) and 56.2% (P< 0.0005, day 16) decrease in melanin content in Melanoderm treated with chloroquine and a 19.8% (day 13) and 42.2% (P< 0.001, day 16) decrease with quinine salicylate treatment, compared to the vehicle-treated tissues. Arbutin did not exhibit as stronger effect on the melanin content of treated MEL-A. Quantitative analysis indicated only a 17.2% (day 13) and 26.8% (P~ 0.05, day 16) decrease.
in melanin content of treated MEL-A. None of the treatments appeared to promote a significant redistribution of melanin pigment in MEL-A (Fig. 3C).

**Chloroquine Inhibits Pigmentation in a Three-dimensional African-American Human Skin Equivalent Model.** Similar experiments were performed on African-American skin equivalents (MEL-B) except that the treatment period was either 10 or 13 days due to the much higher level of melanin deposition in this system. A considerable decrease in relative melanin content of MEL-B was only observed upon topical treatment with 0.5 mM chloroquine for 13 days (Fig. 4A). Only a 20% decreased melanin content was observed in MEL-B treated with 0.5 mM chloroquine for 10 days. However, a 40-50% decrease in melanin content was observed in the MEL-B treated with 0.5 mM chloroquine for 13 days. Quinine salicylate, a less effective pigmentation inhibitor on MEL-A, did not have a substantial effect on melanogenesis of MEL-B (Fig. 4A). Arbutin treatment did not significantly decrease the melanin content of MEL-B (data not shown). Both light microscopic images and Fontana-Masson stained histological results indicated that, consistent with the melanin assay data, chloroquine was the most potent melanogenic inhibitor found to be active on MEL-B (Fig. 4B and 4C). Similar quantitative analysis was performed on 10 Fontana-Masson stained histological sections of either vehicle-treated or compound-treated MEL-B. The results indicated a 23.8% (day 10) and 45.5% (P< 0.0005, day 13) decrease in melanin content in Mel-B treated with chloroquine, compared to the vehicle-treated tissues. The quantitative analysis did not indicate any obvious difference in melanin content of MEL-B treated with quinine salicylate, compared to the vehicle-treated MEL-B, which is consistent with the melanin assay results. Arbutin had little effect on the melanin content of treated MEL-B. Quantitative analysis indicated only a 0.4% (day 10) and 10.3% (day 13) decrease in melanin content of MEL-B treated with arbutin, which is not statistically significant.
Discussion

Our laboratory has employed a chemical genetic screening procedure that uses immortalized mouse melanocytes (melan-a cells) in monolayer culture to identify novel compounds and their targets involved in the regulation of mammalian pigmentation (Ni-Komatsu and Orlow, 2007; Ni-Komatsu and Orlow, 2008). In the current study, we further extend our cell-based screening to examine the response of the three-dimensional reconstituted human skin equivalent model, MelanoDerm, to a group of quinoline analogs, in particular 4-substituted amino quinolines which were identified as novel and potent pigmentation inhibitors by screening the Spectrum Collection, a library of natural products and marketed drugs. This group of quinoline compounds inhibited melanin synthesis by altering the intracellular trafficking of tyrosinase gene family proteins in melanocytes. Most importantly, we have identified chloroquine as a potent depigmenting agent on both African-American and Asian human skin equivalent, and quinine salicylate as a whitening agent on Asian human skin equivalent.

Quinolines have been previously used in the treatment of malaria. As weak bases, quinolines are concentrated in acidic cellular compartments such as lysosomes, where they interfere with lysosomal functions. It is generally believed that these compounds raise the pH of acidic vesicles within sensitive malarial parasites (Goodman and Gilman, 1996). However, beyond the alteration of lysosomal pH, the exact mechanism by which quinolines suppress malaria is not well understood. We found that treatment with quinolines affects the lysosomal compartment in melan-a cells, altering the subcellular localization of Lamp-1.

Melanosomes share in common certain biological properties with lysosomes. Previous studies suggest that many melanosomal proteins utilize intracellular trafficking signals and pathways similar to those of lysosomes and other acidic organelles (Raposo and Marks, 2002;
Dell’Angelica, 2003). Quinolines might also alter the pH of melanosomes. Intracellular pH is an important factor in the regulation of tyrosinase function, dramatically affecting tyrosinase enzymatic activity, and it is critical to the sorting of proteins to melanosomes. In melan-a cells, quinoline analogs appear to disrupt the intracellular trafficking of tyrosinase gene family proteins. The majority of these proteins are trapped in Lamp-1 positive perinuclear compartments in treated cells, contributing to the reduction in pigmentation.

It has been reported that the melanosomes in retinal pigment epithelium (RPE) cells are directly affected by chloroquine (Schraermeyer et al., 1999). Following chloroquine treatment of RPE cultured from cattle eyes, the membranes of melanin granules inside RPE fused and formed large clusters and vacuoles (Schraermeyer et al., 1999), a phenomenon similar to what we observed in chloroquine-treated melan-a cells, where Tyr and Tyrp1 were trapped in clusters near the perinuclear region. Electron microscopic analysis of melan-a cells treated with quinoline compounds indicated that, compared with the vehicle-treated control cells in which melanosomes were mostly present in the mature forms (data not shown), the melanosomes in chloroquine- and mefloquine-treated melan-a cells are predominantly of stage I and II (data not shown). However, melanofilament matrix formation appears normal, which is further supported by the fact that quinoline treatment exhibited no effect on either the protein level or subcellular localization of Pmel17, the major structural protein of the melanosome matrix (data not shown).

Human skin equivalent models such as MelanoDerm provide an alternative to animal testing for evaluating the effects of putative bioactive compounds on mammalian pigmentation. MelanoDerm is considered a more physiologically relevant model than melanocytes alone or melanocyte-keratinocyte co-cultures. The three-dimensional model has the advantage of exhibiting an accurate histologic structure and allows the assessment not only of melanin synthesis in melanocytes, but also of pigment translocation and melanin distribution in
keratinocytes. Human skin equivalents have been used to evaluate novel pigmentation modulators and study the regulation of mammalian pigmentation (Yoon et al., 2003; Takeyama et al., 2004; Matsuda et al., 2008). We evaluated the effects of two 4-amino substituted quinolines on melanogenesis in MelanoDerm by measurement of melanin content, light microscopic observation and image analysis of Fontana-Masson stained MelanoDerm. Our results indicates that compared to the widely used pigmentation inhibitor, arbutin, both quinoline compounds are more effective whitening agents on Asian human skin equivalent (MEL-A), and of the three tested compounds, chloroquine is the most potent depigmenting compound and the only melanogenic inhibitor active in human skin equivalent derived from African-American skin (MEL-B). Furthermore, the inhibitory effects were observed without detectable cytotoxicity.

Even though chloroquine, the most potent pigmentation inhibitor tested in our cell-based assay, was also the most effective one on Melanoderm, the concentration of compound applied to MelanoDerm tissues is much higher than that applied to melanocytes in culture by virtue of the difference in sensitivity between these two assay systems. In the melanocyte monolayer culture system, compounds are added directly to the culture media and need only penetrate the cell membrane; while in the human skin equivalent model, compounds are topically applied to the surface of the tissues, so the final concentrations of compounds that permeate through the tissue to reach the melanocytes is expected to be much lower. In addition, there is also a time difference in the length of treatment between melanocytes and MelanoDerm, which is due to the different pigmentation stages of cultured melanocytes and MelanoDerm. Cultured melan-a cells are already pigmented before treatment with the compounds. By contrast, in the case of either MEL-A or MEL-B, it takes at least 7 to 10 days for the tissues to accumulate melanin evident as a progressive darkening during the culture, and the tissue darkening is much more apparent at 10-14 days.
There are limitations to the MelanoDerm model. First, the structure of the skin equivalent lasts only 3 weeks, which prevents any longer term studies. Second, certain other resident skin cells such as Langerhans cells and dermal fibroblasts are not included in MelanoDerm. It has been suggested that those cell may also contribute to the regulation of pigmentation. Testing in MelanoDerm does not obviate the need for further human clinical testing. Notably, many quinolines, including chloroquine and quinine, have a long history of human usage which would be expected to facilitate their clinical development.

Blue-black pigmentation is a rare complication of oral long-term chloroquine therapy (Dupré et al., 1985; Mahler et al., 1986; Goodman and Gilman, 1996). Its nature is uncertain although a rapid chloroquine-induced clumping of pigment and more delayed and subtle pigment changes produced by long-term quinine or mefloquine have been reported (Dupré et al., 1985; Mahler et al., 1986; Goodman and Gilman, 1996). This most likely is due to the high affinity of these compounds for melanin. Hypopigmentation of hair and freckles has also been reported (Dupré et al., 1985; Mahler et al., 1986; Goodman and Gilman, 1996). However, our data indicated that quinolines potently inhibited melanin synthesis in melanocytes. Therefore, in treated skin, there would be less or no melanin for this group of compounds to bind to. In addition, there is a difference between oral administration of quinolines and their topical application. While the concentration of this group of compounds required to treat malaria is much higher than the concentration needed to inhibit melanogenesis, the skin concentration achieved upon oral administration are unknown.

In conclusion, we have identified a series of quinoline compounds as potent pigmentation inhibitors in culture murine melanocytes through a chemical genetic approach. Two of the quinolines, chloroquine and quinine salicylate, inhibited melanogenesis in a three-dimensional human skin equivalent model to a greater degree than arbutin. Our results indicate that
chloroquine and quinine salicylate are potential effective whitening agents for cosmetics and the medical treatment of disorders of pigmentation.
References


Toyofuku K, Wada I, Valencia JC, Kushimoto T, Ferrans VJ, and Hearing VJ (2001) Oculocutaneous albinism types 1 and 3 are ER retention diseases: mutation of tyrosinase or Tyrp1 can affect the processing of both mutant and wild-type proteins. *FASEB J* 15(12): 2149-2161.


Footnotes

The work was supported by NIH grant AR41880 and by Luminessence, Inc. to SJO. SJO is a founder of Luminessence, Inc. and SJO and LNK are minority shareholders in Luminessence, Inc.. ChunXiang Tong, Guangming Chen and Nelya Brindzei state no conflict of interest.
Figure Legends

**Fig. 1.** Quinolines alter the intracellular trafficking of tyrosinase (Tyr) and tyrosinase-related protein 1 (Tyrp1).  
A, Alterations of the subcellular localization of Tyr and Tyrp1 by quinolines. Immunofluorescence confocal microscopy images are shown with immunostaining of Tyr (green) and Tyrp1 (red). The colocalization of tyrosinase with tyrosinase-related protein 1 is indicated in yellow in the merged image. Scale bars, 5 μm.  
B, Tyrosinase trafficks beyond the TGN in melan-a cells treated with quinolines. Immunofluorescence confocal microscopy images are shown with immunostaining of Tyr (green) and TGN38 (red). The colocalization of tyrosinase with TGN38 is indicated as yellow in the merged image. Scale bars, 5 μm.

**Fig. 2.** Quinolines induce partial colocalization of tyrosinase and Lamp-1. Immunofluorescence confocal microscopy images are shown with immunostaining of Tyr (red) and Lamp-1 (green). The colocalization of tyrosinase with Lamp-1 is indicated as yellow in the merged image. Scale bars, 5 μm.

**Fig. 3.** Chloroquine and quinine salicylate decrease melanin accumulation of Asian human skin equivalent (MEL-A). MEL-A were treated with 0.5 mM chloroquine, 4.0 mM quinine salicylate, 3 mg/ml arbutin or controls (either water or 30% water: 70 % PEG) for 13 or 16 days. Representative data from multiple experiments are presented.  
A, Changes in melanin content of MEL-A. Data are presented as the percentage of control, and each column represents the mean ± SD of multiple experiments.  
B, Phase-contrast microscopic images of MEL-A.  
C, Immunohistochemical appearances of MEL-A after Fontana-Masson staining to emphasize melanin content.

**Fig. 4.** Chloroquine decrease melanin accumulation of African-American human skin equivalent (MEL-B). MEL-B were treated with 0.5 mM chloroquine, 4.0 mM quinine salicylate, 3 mg/ml
arbutin or controls (either water or 30% water: 70% PEG) for 10 or 13 days. Representative data from multiple experiments are presented. A, Changes in melanin content of MEL-B. Data are presented as the percentage of control, and each column represents the mean ± SD of multiple experiments. B, Phase-contrast microscopic images of MEL-B. C, Immunohistochemical appearances of MEL-B after Fontana-Masson staining to emphasize melanin content.
Table 1. Structures of quinoline analogs that inhibit pigmentation and their IC\textsubscript{50} values on melanin synthesis in melan-a cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC\textsubscript{50} ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amodiaquine dihydrochloride</td>
<td><img src="image" alt="Amodiaquine" /></td>
<td>2.6 ± 0.15</td>
</tr>
<tr>
<td>Chloroquine</td>
<td><img src="image" alt="Chloroquine" /></td>
<td>0.7 ± 0.18</td>
</tr>
<tr>
<td>Cinchonidine</td>
<td><img src="image" alt="Cinchonidine" /></td>
<td>10.9 ± 0.22</td>
</tr>
<tr>
<td>Dibucaine hydrochloride</td>
<td><img src="image" alt="Dibucaine" /></td>
<td>10.2 ± 0.28</td>
</tr>
<tr>
<td>Mefloquine hydrochloride</td>
<td><img src="image" alt="Mefloquine" /></td>
<td>2.0 ± 0.15</td>
</tr>
<tr>
<td>Primaquine diphosphate</td>
<td><img src="image" alt="Primaquine" /></td>
<td>6.8 ± 0.37</td>
</tr>
<tr>
<td>Quinacrine hydrochloride</td>
<td><img src="image" alt="Quinacrine" /></td>
<td>1.8 ± 0.17</td>
</tr>
<tr>
<td>Quinidine</td>
<td><img src="image" alt="Quinidine" /></td>
<td>2.7 ± 0.14</td>
</tr>
<tr>
<td>Quinine</td>
<td><img src="image" alt="Quinine" /></td>
<td>1.6 ± 0.15</td>
</tr>
<tr>
<td>Quinine ethyl carbonate</td>
<td><img src="image" alt="Quinine ethyl carbonate" /></td>
<td>5.1 ± 0.12</td>
</tr>
<tr>
<td>Quinine salicylate</td>
<td><img src="image" alt="Quinine salicylate" /></td>
<td>3.6 ± 0.28</td>
</tr>
</tbody>
</table>
### Table 2. Structures of cinchona alkaloids and quinolines and their effects on melanin synthesis in melan-a cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>% inhibition at 1 μM</th>
<th>% inhibition at 5 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinchonidine</td>
<td><img src="image1" alt="Structure" /></td>
<td>25.0</td>
<td>35.7</td>
</tr>
<tr>
<td>Hydrocinchonine</td>
<td><img src="image2" alt="Structure" /></td>
<td>24.0</td>
<td>37.3</td>
</tr>
<tr>
<td>Hydroquinidine</td>
<td><img src="image3" alt="Structure" /></td>
<td>30.9</td>
<td>52.3</td>
</tr>
<tr>
<td>Hydroquinidine 4-chlorobenzoate</td>
<td><img src="image4" alt="Structure" /></td>
<td>10.0</td>
<td>25.5</td>
</tr>
<tr>
<td>Hydroquinine</td>
<td><img src="image5" alt="Structure" /></td>
<td>30.5</td>
<td>67.5</td>
</tr>
<tr>
<td>Hydroquinine hydrobromide hydrate</td>
<td><img src="image6" alt="Structure" /></td>
<td>26.8</td>
<td>67.4</td>
</tr>
<tr>
<td>N-Benzylcinchonidinium chloride</td>
<td><img src="image7" alt="Structure" /></td>
<td>9.6</td>
<td>16.4</td>
</tr>
</tbody>
</table>
B Figure 1B

<table>
<thead>
<tr>
<th></th>
<th>Tyr</th>
<th>TGN38</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><img src="image" alt="Control Tyr" /></td>
<td><img src="image" alt="Control TGN38" /></td>
<td><img src="image" alt="Control Merge" /></td>
</tr>
<tr>
<td>Chloroquine</td>
<td><img src="image" alt="Chloroquine Tyr" /></td>
<td><img src="image" alt="Chloroquine TGN38" /></td>
<td><img src="image" alt="Chloroquine Merge" /></td>
</tr>
<tr>
<td>Quinidine</td>
<td><img src="image" alt="Quinidine Tyr" /></td>
<td><img src="image" alt="Quinidine TGN38" /></td>
<td><img src="image" alt="Quinidine Merge" /></td>
</tr>
<tr>
<td>Quinine</td>
<td><img src="image" alt="Quinine Tyr" /></td>
<td><img src="image" alt="Quinine TGN38" /></td>
<td><img src="image" alt="Quinine Merge" /></td>
</tr>
<tr>
<td>Quinine salicylate</td>
<td><img src="image" alt="Quinine salicylate Tyr" /></td>
<td><img src="image" alt="Quinine salicylate TGN38" /></td>
<td><img src="image" alt="Quinine salicylate Merge" /></td>
</tr>
</tbody>
</table>
Figure 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tyr</th>
<th>Lamp-1</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><img src="control.png" alt="Image" /></td>
<td><img src="control_lamp-1.png" alt="Image" /></td>
<td><img src="control_merge.png" alt="Image" /></td>
</tr>
<tr>
<td>Chloroquine</td>
<td><img src="chloroquine.png" alt="Image" /></td>
<td><img src="chloroquine_lamp-1.png" alt="Image" /></td>
<td><img src="chloroquine_merge.png" alt="Image" /></td>
</tr>
<tr>
<td>Quinidine</td>
<td><img src="quinidine.png" alt="Image" /></td>
<td><img src="quinidine_lamp-1.png" alt="Image" /></td>
<td><img src="quinidine_merge.png" alt="Image" /></td>
</tr>
<tr>
<td>Quinine</td>
<td><img src="quinine.png" alt="Image" /></td>
<td><img src="quinine_lamp-1.png" alt="Image" /></td>
<td><img src="quinine_merge.png" alt="Image" /></td>
</tr>
<tr>
<td>Quinine salicylate</td>
<td><img src="quinine_salicylate.png" alt="Image" /></td>
<td><img src="quinine_salicylate_lamp-1.png" alt="Image" /></td>
<td><img src="quinine_salicylate_merge.png" alt="Image" /></td>
</tr>
<tr>
<td>Mefloquine hydrochloride</td>
<td><img src="mefloquine.png" alt="Image" /></td>
<td><img src="mefloquine_lamp-1.png" alt="Image" /></td>
<td><img src="mefloquine_merge.png" alt="Image" /></td>
</tr>
</tbody>
</table>
B Figure 4B

Day 10  Day 13

Control

Chloroquine

Arbutin

Day 10  Day 13

Control

Quinine salicylate