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Inhibitors of Mammalian Melanocyte Tyrosinase: *In Vitro* Comparisons of Alkyl Esters of Gentisic Acid with Other Putative Inhibitors

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ABSTRACT. To discover safe and effective topical skin-lightening agents, we have evaluated alkyl esters of the natural product gentisic acid (GA), which is related to our lead compound methyl gentisate (MG), and four putative tyrosinase inhibitors, utilizing mammalian melanocyte cell cultures and cell-free extracts. Desirable characteristics include the ability to inhibit melanogenesis in cells ($IC_{50} < 100$ μg/mL) without cytotoxicity, preferably due to tyrosinase inhibition. Of the six esters synthesized, the smaller esters (e.g. methyl and ethyl) were more effective enzyme inhibitors ($IC_{50} \sim 11$ and 20 μg/mL, respectively). For comparison, hydroquinone (HQ), a commercial skin "bleaching" agent, was a less effective enzyme inhibitor ($IC_{50} \sim 72$ μg/mL), and was highly cytotoxic to melanocytes *in vitro* at concentrations substantially lower than the IC_{50} for enzymatic inhibition. Kojic acid was a potent inhibitor of the mammalian enzyme ($IC_{50} \sim 6$ μg/mL), but did not reduce pigmentation in cells. Both arbutin and magnesium ascorbyl phosphate were ineffective in the cell-free and cell-based assays. MG at 100 μg/mL exhibited a minimal inhibitory effect on DHICA oxidase (TRP-1) and no effect on DOPAchrome tautomerase (TRP-2), suggesting that MG inhibits melanogenesis primarily via tyrosinase inhibition. MG and GA were non-mutagenic at the *hprt* locus in V79 Chinese hamster cells, whereas HQ was highly mutagenic and cytotoxic. The properties of MG *in vitro*, including (1) pigmentation inhibition in melanocytes, (2) tyrosinase inhibition and selectivity, (3) reduced cytotoxicity relative to HQ, and (4) lack of mutagenic potential in mammalian cells, establish MG as a superior candidate skin-lightening agent. BIOCHEM PHARMACOL **57**;6:663–672, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. melanogenesis; TRP-1; TRP-2; hydroquinone; arbutin; kojic acid; magnesium ascorbylphosphate; cosmeceutical; methyl gentisate; pigmentation

Melanogenesis is the process of production (and subsequent distribution) of melanin by melanocytes within the skin and hair follicles [1, 2]. Melanocytes have specialized lysosome-like organelles, termed melanosomes, which contain several enzymes that mediate the production of melanin. The copper-containing enzyme tyrosinase (a tyrosine hydroxylase; EC 1.14.18.1) catalyzes the oxidation of the amino acid tyrosine into DOPA‖ and subsequently DOPAquinone. At least two additional melanosomal enzymes are involved in the eumelanogenesis pathway that produces brown and black pigments, including TRP-1 (DHICA oxidase), and TRP-2 (DOPAchrome tautomerase). Depending on the incorporation of a sulfur-containing reactant (e.g. cysteine or glutathione) into the products, the melanogenesis pathway diverges, producing eumelanins or pheomelanins (amber and red pigments) or both.

Various dermatologic disorders result in the accumulation of excessive levels of epidermal pigmentation. These hyperpigmented lentigenes include melasma, age spots or liver spots, and sites of actinic damage (i.e. due to solar ultraviolet irradiation; [3]). Current therapies are inadequate for these conditions. Vitiligo is a hyperpigmentary condition of unknown etiology, in which melanocyte destruction results in regional losses of pigmentation. Potentially, it can be treated by pharmacologic depigmentation of the surrounding unaffected area of skin. Furthermore, a global market demand has developed recently for skin-lightening agents as "vanity" cosmeceutical products, because lighter skin color is preferred by some dark-skinned individuals in many countries and races [4, 5]. Unfortunately, several purportedly active agents (e.g. arbutin and kojic acid, among others) have not been demonstrated yet to be clinically efficacious when critically analyzed in carefully controlled studies. The U.S. FDA-approved phar-

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‖ *Abbreviations:* DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DOPA, 3,4-dihydroxyphenylalanine; GA, gentisic acid; HQ, hydroquinone; MG, methyl gentisate; OTC, over-the-counter; TRP-1, tyrosinase-related protein-1; TRP-2, tyrosinase-related protein-2; FAB, fast atom bombardment; and MAP, magnesium L-ascorbyl-2-phosphate

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maceutical products containing 2–4% HQ are moderately efficacious, but HQ is considered to be cytotoxic to melanocytes and potentially mutagenic to mammalian cells [3–5; this work].

We previously discovered that MG, the methyl ester of GA (2,5-dihydroxybenzoic acid), is an inhibitor of melanin accumulation in a murine melanocyte cell culture primary screen [6]. GA is a natural product from the root of the genus *Gentiana*, named after Gentius, an Illyrian (Greco-Roman) king of the 2nd century B.C., said to have first discovered the medicinal properties of the plant [7]. The sodium salt of GA is thought to be an analgesic and an anti-inflammatory agent. GA is a ubiquitous metabolite, produced not only by plants, but also by *Penicillium patulum* and *Polyporus tumulosus*, and is excreted into the urine of mammals following ingestion of salicylates [8, 9]. We have not determined yet whether the methyl ester is a natural product, although we anticipate that it is likely to occur.

MG and GA are structurally similar to HQ (see Fig. 2). HQ has been reported to induce mutations in *Salmonella* and at the *hprt* locus of treated Chinese hamster V79 cells [10, 11]. HQ appears to be an important intermediate in the bioactivation of the carcinogen benzene [12]. The mutagenic potential of MG and GA is examined here for the first time, using the same conventional mammalian cell culture system in which HQ showed mutagenic effects.

A general strategy was described recently by one of us for the discovery and development of novel topical skin-lightening products [5]. Desirable skin-lightening agents inhibit the synthesis of melanin in melanosomes by acting specifically to reduce the synthesis or activity of tyrosinase, exhibit low cytotoxicity, and are non-mutagenic. A refined triple-endpoint methodology is presented here for the discovery and assessment of such agents. Newly synthesized alkyl esters of GA and four putative inhibitors of tyrosinase were screened and compared.

MATERIALS AND METHODS

Synthesis of the Alkyl Gentisate Series

Methyl, ethyl, *n*-propyl, *i*-propyl, *n*-butyl, and *n*-pentyl esters of GA were prepared by refluxing GA (Aldrich Chemical Co.) in an excess of the corresponding alcohol for 2–7 days, using *p*-toluenesulfonic acid as a catalyst. The 2-methoxyethanol ester of GA was prepared by refluxing stoichiometric amounts of the methoxyethanol ester and GA in dioxane for 14 days, again using *p*-toluenesulfonic acid as a catalyst. The courses of the reactions were followed by TLC, quenching the reactions when they appeared to be stalled. Work-up included evaporation of the solvent followed by extraction with ethyl acetate, water, and small aliquots of aqueous sodium bicarbonate until no GA was visible in the organic phase by TLC. The desired esters were obtained by drying the ethyl acetate solutions over sodium sulfate followed by evaporation to dryness. Product structures were verified by ^{1}H NMR, MS (FAB), and elemental analysis. For comparison, MG was also obtained commercially from the Apin Chemicals Co.

Compound Testing in Cultured MelAb Cell Assays

Compound testing *in vitro* was performed using the protocol of Dooley and coworkers [6], with minor modifications. The cellular-based assays were performed in triplicate at least twice. On day 1, a total of 10^5 MelAb cells were added to each well of the 24-well plates, and were incubated at 37° in 5% CO_2. On day 2, concentrated stock solutions were prepared of test compounds at 20 mg/mL in "vehicle" (50% propylene glycol, 30% ethanol, 20% water), followed by incubation at 55° for 30 min to dissolve the compounds. Serial dilutions of each stock solution were prepared at concentrations ranging from 2000 to 0.64 μg/mL, in 10% vehicle/90% PBS, by attenuating the concentrated stocks 10-fold in PBS solution. Solutions were exposed to cells on day 2 in triplicate wells at final concentrations ranging from 200 to 0.064 μg/mL (1% vehicle, 9% PBS, 90% medium final concentration). On day 4, the cells were examined using an inverted phase microscope for viability. On day 6 (following 4 days of agent exposure), the assays were terminated by replacing the medium with PBS.

Determination of Melanin Content

Melanin content was determined using the published protocol [6] with minor modifications. The cytotoxicity assays were performed in triplicate at least twice. After washing the cells with PBS, we decanted the wash by inversion and emptied the wells by tapping the inverted plates on paper towels. The cells were lysed with 1 mL of 1 N NaOH and pipetted repeatedly to homogenize. For analysis, 200 μL of each crude cell extract were transferred into 96-well plates. Relative melanin content was determined by optical density at 405 nm using a SpectramaxPlus spectrophotometer.

Quantitation of Adherent Cell Number by Crystal Violet Staining

Quantitation of cell number (to determine whether compounds are cytotoxic/cytostatic) was obtained following the protocol of Dooley *et al.* [6], except for minor modifications. After washing the cells with PBS and emptying the wells by inversion and tapping the inverted plates against paper towels, 0.5 mL of 0.1% crystal violet in 10% ethanol was added to each well. After staining the wells for 5 min at room temperature, the stain was decanted by inversion, and the plates were rinsed four times in beakers of water. After rinsing, the plates were emptied once again as described. To extract crystal violet from the cells, 1 mL of 95% ethanol was added to each well, and the plates were placed on a rotating platform for 20+ min. For analysis, 100 μL of solution was transferred from each well into appropriate wells in 96-well plates. To determine the relative number of cells in each well, we measured the absorption by crystal

violet in the solutions at 540 nm using a SpectramaxPlus spectrophotometer.

Tyrosinase Enzyme Assay

To screen compounds for potential to inhibit mammalian tyrosinase, cell lysates were prepared and used according to the protocol by Hearing [1] with modifications. The enzyme activity determinations were performed in duplicate at least twice. Approximately 10^6 MelAb (immortalized mouse melanocyte) cells were detached from a 10-cm culture plate using a cell scraper, and then were centrifuged and resuspended in 6 mL PBS. The cells were lysed by freeze–thaw cycling the tube five times, by transferring it between a dry-ice ethanol bath and a water bath at room temperature. After the final thawing, the cell suspension was disrupted using a glass homogenizer, and lysis was confirmed using a microscope. Samples of about 1.5 mL were aliquoted and stored at −80° until required.

One day prior to compound testing, we prepared 1 L of "Wash" solution (0.1 N HCl), 1 L of "Stop" solution (1 mM L-tyrosine in 0.1 N HCl), and 100 mL of vehicle solution (50% propylene glycol, 30% ethanol, 20% water). Serial dilutions of test compounds were prepared at 5× stock concentrations ranging from 1000 to 1.6 μg/mL (in 5% vehicle, 95% PBS), and stored at -20° overnight. On the day of the assay, we prepared 100 mL of "Activator" solution (0.5 mM l-DOPA in PBS) and 5 mL "Substrate" solution (125 μL of 0.5 mM [U-^{14}C]L-tyrosine at 50 μCi/mL in 0.1 N HCl added to 4.875 mL of 0.5 mM L-tyrosine in PBS). A single 10-cm culture plate of MelAb cells provides sufficient cell-free "tyrosinase" extract to Test 16 compounds in two 96-well plates.

Tyrosinase assays were performed in 96-well plates, with 10 μL of "Inhibitor" per well. For the negative control, 30 μL of PBS was added, and for the positive control, 10 μL of 5% vehicle/95% PBS. After blending 20 μL of "Tyrosinase" solution into all wells (except for the PBS control) by repeated pipetting, these mixtures were preincubated for 30 min at room temperature. Enzymatic reactions were started by adding 10 μL of "Substrate" solution and 10 μL of "Activator" solution into all wells, yielding final concentrations ranging from 200 to 0.32 μg/mL (in 1% vehicle/99% PBS). The plates were incubated at 37° for 2 hr, and terminated by adding 100 μL "Stop" solution to each well.

The reactions were transferred to a Zeta Probe nylon blotting membrane (Bio-Rad), in a 96-well Schleicher & Schuell dot blotter, reinforced below with 2 sheets of Whatman 3MM filter paper. Solutions were allowed to bind for 15 min at room temperature, before vacuum filtration through the membrane until dry. Wells were washed three times with 250 μL of "Stop" solution. The membranes were washed within a plastic box, in 100 mL of "Wash" solution 3 × 20 min, rotating gently on an orbit shaker, and were air-dried overnight. Then, the ^{14}C decay was monitored using an AMBIS plate reader for 2 hr, and the results were quantified using AMBIS software.

Determinations of IC_{50} Values

A first order exponential equation was fit to the data to obtain the 50% inhibition concentration (IC_{50}) values for each assay. The "Physica" program package [13] was run on a Silicon Graphics work station. By fitting the equation $I = I_0 + I_1\exp(-C/C_i)$ to each of the data sets, the IC_{50} values of each titration series were obtained. By definition: I = intensity of measured variable at compound concentration C; I_0 = estimated background intensity constant; I_1 = estimated total intensity change in measured variable; $I_0 + I_1$ = maximum total variable intensity (the negative control intensity); C = compound concentration; and C_i = estimated compound inhibition constant, such that $C_i \ln(2) = IC_{50}$ of the compound. Mean deviations of each data point were calculated along with standard deviations of the various IC_{50} values.

Melanogenesis Enzymatic Profile

To assay the mechanism of action of MG against the three major melanogenesis enzymatic activities (i.e. tyrosinase, DOPAchrome tautomerase, and DHICA oxidase), melanocyte cell extracts were prepared from mouse Melan-a cells (from which the MelAb line was derived), provided by Dr. D. Bennett [14]. The cellular lysates were prepared using NP-40 and SDS, and all enzymatic assays were performed at pH 6.8, 37° for 60 min. The test compounds (MG and GA) were solubilized in ethanol. Various melanogenesis enzymatic assays were performed (listed below). The multi-enzyme profile characterization of MG was performed at 100 μg/mL (595 μM) for each assay, a concentration that is significantly higher than the known IC_{50} for depigmenting activity on MelAb cells [6], equivalent to our arbitrary threshold for activity in cultured cells [6], and similar to the reported apparent K_m(600 μM) for tyrosine as the substrate on mouse tyrosinase [15].

(A) Tyrosinase. Tyrosine hydroxylase activity was determined by two methods. The first monitored the production of tritiated H_2O from the hydroxylation of L-[3,5-^{3}H]tyrosine (Dupont-NEN) to DOPA [1]. This is referred to as the "direct" method. A second and "indirect" method to determine tyrosinase activity utilized the [^{14}C]tyrosine (Dupont-NEN) incorporation assay [1], described above for compound screening.

(B) DOPAchrome tautomerase (TRP-2). Inhibition of enzyme activity by 100 μg/mL of MG was assessed using DOPAchrome as the substrate. The disappearance of the substrate and the production of DHICA were monitored by HPLC [16]. DOPAchrome was prepared using the silver oxide method [17], and DHICA as a standard was provided by Professor S. Ito.

(C) DHICA oxidase (TRP-1). Inhibition of enzyme activity was determined using the method of Kobayashi and

coworkers [18], with immunopurified DHICA oxidase and 100 μg/mL of MG.

Mammalian V79 Mutagenicity Assays

Chinese hamster V79 cells (subclone V79-MZ) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL), at 37° in a humidified atmosphere containing 5% CO_2. The protocol used for determination of mutagenicity was identical to that used routinely in previous studies [10, 11, 19–21]. A total of 1.5×10^6 cells and 30 mL medium were added to each 15-cm Petri dish. After 18 hr, the test compound was added using 60 μL DMSO as the solvent. The exposure was terminated by changing the medium 24 hr later. After an expression period of 6 days with one subculture, cells were replated at a density of 1×10^6 per 15-cm Petri dish in medium containing 6-thioguanine (7 μg/mL) for the selection of mutants (six dishes), or at a density of 100 cells/9-cm Petri dish in medium without 6-thioguanine for the determination of the cloning efficiency (three dishes). The cultures were fixed and stained, and the colonies were counted after about 10 days. Mutant frequencies were calculated (6-thioguanine-resistant colonies per 1×10^6 plated viable cells). For the evaluation of the results, empiric criteria were used. A compound was considered to be negative in the test if the mutant frequencies of all treatment groups were less than the mean value of the solvent control cultures plus 10×10^{-6}. It was considered positive if the mutant frequency was at least three times above the value of the solvent control cultures and at least 20×10^{-6}.

The cytotoxicity in the mutagenicity test was determined by counting the cells harvested at the subcultivation during the expression period [20]. When the cell density was optically indistinguishable from that of the solvent control, the cells were not always counted. From numerous other experiments we estimate that the cell numbers are at least 85% of the control value. The cells also were not counted when their density was so low that an appropriate determination of the mutant frequency was precluded. This corresponds to a cell number of less than 2–5% of the control value.

RESULTS

Enzymatic Specificity of MG

To demonstrate that MG is a tyrosinase inhibitor and to discern whether any other of the key melanosomal enzymes implicated in the production of melanin were the molecular targets of MG action, biochemical assays were performed on each of the key enzymes either from cellular extracts or from purified proteins. Three enzymes (tyrosinase, DOPAchrome tautomerase, and DHICA oxidase) were profiled for inhibition by MG at a high concentration of 100 μg/mL (595 μM), at levels substantially higher than

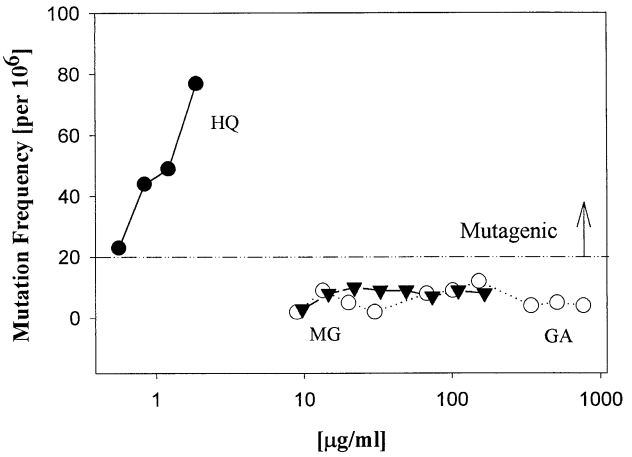

FIG. 1. Mammalian V79 cell mutagenicity results. The frequencies of mutations (number of colonies × 10^{-6}) at the *hprt* locus in Chinese hamster V79 cells are plotted versus the μg/mL concentrations of the test agents (HQ, ●; MG, ▼; and GA, ○). Concentrations were titrated in cultures of 1×10^6 cells to the point of cytotoxicity. Frequencies above 20×10^{-6} are considered mutagenic. This titration is representative of three experiments.

required to inhibit melanogenesis in cultured cells [6]. This concentration is equivalent to the apparent K_m for tyrosine as a substrate on mouse tyrosinase (600 μM) [15]. Tyrosinase activity was determined by two different radiochemical methods [1], either "directly" using [^{3}H]tyrosine in a tyrosine hydroxylase assay, or "indirectly" using the [^{14}C]tyrosine incorporation assay. In repeat experiments, this enzyme from mouse melanocyte extracts (purified enzyme) was inhibited effectively by 100 μg/mL of MG, as expected, whereas at the same high dose DOPAchrome tautomerase was not affected, and DHICA oxidase was inhibited only minimally (retained 76% of control). At this concentration, tyrosinase activity was reduced to less than 25% of vehicle control in numerous experiments (data not shown). Unlike the activity observed for MG, GA was ineffective ($IC_{50} > 600$ μg/mL) as a tyrosinase enzyme inhibitor (data not shown).

Mutagenicity Assays

HQ, MG, and GA were tested in mammalian V79 cell mutagenesis assays, and the results are presented in Fig. 1. The standard positive control compound, *anti*-chrysene-1,2-dihydrodiol-3,4-oxide, showed strong mutagenic effects. This potent control produced a mutant frequency of 825×10^{-6} at a concentration of 3.6 μM, which was similar to the effects observed previously [19]. An unambiguous mutagenic effect was detected with HQ as expected, although the effect was somewhat weaker and required higher concentrations than those in the previous studies [10, 11]. Under the same conditions, the mutant frequencies in the MG- and GA-treated cultures ($2–13 \times 10^{-6}$) remained within the range observed in the control cultures ($2–15 \times 10^{-6}$). In addition, both compounds were

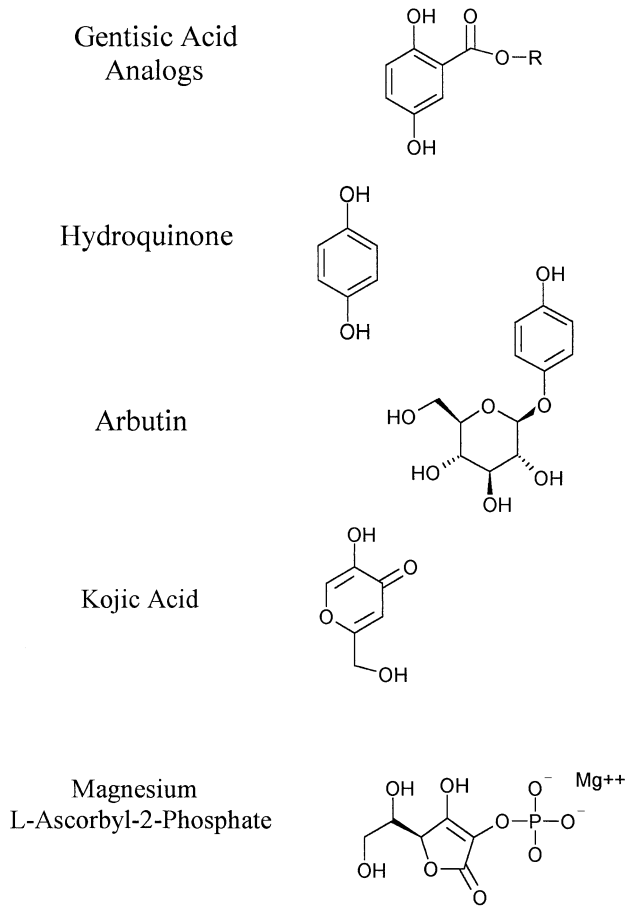

FIG. 2. Putative topical skin-lightening agents: Chemical structures of putative, topical skin-lightening agents. Analogs of gentisic acid (GA), R = H, include the alkyl ester series: R = CH_3, methyl; CH_2CH_3, ethyl; $(CH_2)_2CH_3$, *n*-propyl; $CH(CH_3)_2$, *i*-propyl; $(CH_2)_3CH_3$, *n*-butyl; $(CH_2)_4CH_3$, *n*-pentyl; $(CH_2)OCH_3$, methoxy-ethyl. Hydroquinone (HQ) is a skin bleaching agent, and arbutin is a glycosylated "prodrug" form of hydroquinone. Kojic acid is a copper-chelating agent. Magnesium L-ascorbyl-2-phosphate is an antioxidant.

substantially less cytotoxic than HQ. The cytotoxic LC_{50} values of HQ, MG, and GA in V79 cells were approximately 2.2, 134, and 771 μg/mL (or 20, 800, and 5000 μM, respectively). Concentrations that produced <5% cell viability were not included in the analysis. Thus, HQ was approximately forty times more toxic than MG in these mammalian cells, in addition to being highly mutagenic, whereas MG was not mutagenic at any concentration.

Purported Tyrosinase Inhibitors

We tested four commercially available, putative skin-lightening agents and compared them with MG in our three primary screens. Structures of these compounds are shown in Fig. 2. The third column of Table 1 presents tyrosinase enzyme IC_{50} values for these compounds. Kojic acid and MG were the most potent "free" enzyme inhibitors, with IC_{50} values of *ca.* 6 and 11 μg/mL, respectively. HQ and arbutin were relatively poor enzyme inhibitors, with IC_{50} values of about 72 and 149 μg/mL, respectively. Magnesium ascorbylphosphate (MAP) did not inhibit tyrosinase enzyme in these assays, even at 200 μg/mL.

Column 4 of Table 1 presents IC_{50} values for inhibition of cultured MelAb melanocyte pigmentation (due to both the amounts of melanin synthesis and total cell number) for these same compounds. HQ was a very effective agent with an IC_{50} of about 1 μg/mL, albeit due to cytotoxicity. Here MG was an effective pigmentation inhibitor, with an IC_{50} of about 31 μg/mL, falling within the range of numerous prior similar experiments (range of *ca.* 15–60 μg/mL). The other compounds in this series did not inhibit pigmentation in intact melanocytes at concentrations up to 200 μg/mL.

Column 5 of Table 1 shows MelAb cell cytotoxicity IC_{50} values for these compounds. MG was relatively non-toxic, with an IC_{50} of cytotoxicity/cytostasis of about 119 μg/mL on MelAb cells. HQ was extremely toxic; it had an IC_{50} value of about 3 μg/mL in this assay. The other commercial compounds were non-toxic to MelAb cells up to 200 μg/mL.

Based upon our experience with over 150 compounds in this assay system [5, 6; this work; and unpublished results], we arbitrarily define a potentially efficacious skin depigmentation candidate agent as one that inhibits tyrosinase enzyme with an IC_{50} of <25 μg/mL, inhibits melanocyte cell pigmentation with an IC_{50} < 100 μg/mL, and is non-cytotoxic to cells with an IC_{50} > 100 μg/mL. The threshold criteria are based upon: (1) our published stan-

TABLE 1. *In vitro* assessments of putative skin-lightening agents

Compound	Relative mass [MG]	Cell-free tyrosinase IC_{50} (μg/mL)	Melanocyte pigmentation IC_{50} (μg/mL)	Melanocyte cytotoxicity IC_{50} (μg/mL)	Assessment
Desired agent		**<25**	**<100**	**>100**	Effective
HQ	0.655	72 ± 25	**1.1 ± 0.2**	3.1 ± 0.7	Toxic
MG	1.000	**11.2 ± 4**	**30.9 ± 5**	**118.7 ± 12**	Effective
Kojic acid	0.845	**6.2 ± 2**	>200	**>200**	Ineffective
Arbutin	1.62	149 ± 41	>200	**>200**	Ineffective
MAP	1.68	>200	>200	**>200**	Ineffective

Experimental *in vitro* evaluations of putative skin-lightening agents, using tyrosinase enzyme, melanogenesis, and cytotoxicity assays, are displayed along with an overall assessment. The relative mass of each compound is denoted with respect to MG. Based on the characteristics of MG and our experience with >150 compounds, we designate a desired candidate as one that inhibits by 50% (IC_{50}) mammalian tyrosinase enzyme at concentrations < 25 μg/mL, inhibits pigmentation in mammalian melanocytes < 100 μg/mL, and exhibits low cytotoxicity at concentrations > 100 μg/mL. Magnesium ascorbyl phosphate is denoted as MAP.

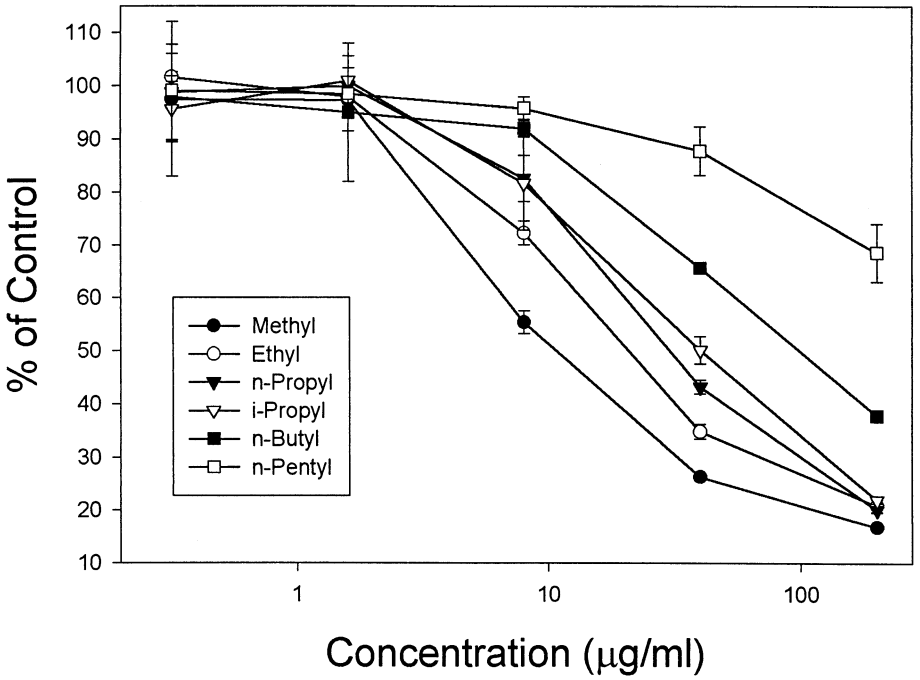

FIG. 3. Cell-free tyrosinase assay: A representative titration assay of the inhibition of murine tyrosinase in cell-free lysates, by alkyl esters of GA. Tyrosinase enzyme converts [^{14}C]L-tyrosine into [^{14}C]L-DOPA and subsequently into [^{14}C]L-DOPAquinone. The labeled reaction products bind to a nylon filter (in a 96-well dot blotter apparatus), while unconverted tyrosine is washed away. Relative product (bound [^{14}C]L-DOPAquinone) was measured using an AMBIS radiometric plate reader, and results are displayed as percent of vehicle control (lacking testing agent) as a function of inhibitor concentration (100% represents *ca.* 2400 counts in 2 hr per well). The results of duplicate samples are expressed as the average ± range, plotted using SigmaPlot. These experiments were repeated twice.

dard (i.e. 100 μg/mL) for cell-based assays [6]; (2) a more stringent standard for free enzyme (i.e. 25 μg/mL), because the agents must pass through two cellular membranes to inactivate the enzyme within melanosomes in cultured cells, and optimal uptake is not necessarily assured; and (3) our general experience in screening many phenolic compounds in the various assays. Of the compounds tested in this current study, only MG satisfied all of these requirements. Based on these multiple *in vitro* endpoints, it is the best candidate skin-lightening agent that we are aware of from the literature (and this work), and its behavior in these assays establishes the standards for further rational drug design to identify additional comparable or better active agents. HQ is highly toxic and mutagenic [this work; 6, 10, 11, 22]. Other purportedly active compounds tested in this series were non-efficacious. They did not act on MelAb cells or the enzyme, or both. Our assessments of these compounds are denoted in the last column of Table 1.

Inhibition by Alkyl Esters of GA

Having established that MG is the superior candidate among the purported skin-lightening agents, we sought to expand upon our understanding of its mechanism by performing a limited structure–activity relationship (SAR) study. Results for a representative cell-free screen of tyrosinase inhibition by our alkyl ester series are presented in Fig. 3. The figure shows a systematic diminution in the ability to inhibit tyrosinase by gentisate compounds with increasing size of the alkyl ester moiety.

Table 2 presents results for the alkyl esters in all three assays. The values given are averages for two separate titration assays. Column 3 lists tyrosinase IC_{50} values of these compounds, obtained using our cell-free enzymatic assay. Gentisate compounds with smaller ester moieties inhibited the enzyme more effectively than those with larger ester moieties. MG was the most effective inhibitor of

TABLE 2. *In vitro* assessments of alkyl esters of GA

R group	Relative mass	Cell-free tyrosinase IC_{50} (μg/mL)	Melanocyte pigmentation IC_{50} (μg/mL)	Melanocyte cytotoxicity IC_{50} (μg/mL)
Methyl	1.00	**11.2 ± 4**	**30.9 ± 5**	**118.7 ± 12**
Ethyl	1.08	**19.6 ± 7**	**28.7 ± 5**	65.9 ± 20
n-Propyl	1.17	28.5 ± 12	**15.6 ± 2**	56.5 ± 10
i-Propyl	1.17	57.5 ± 21	**27.8 ± 8**	62.7 ± 9
n-Butyl	1.25	128 ± 53	**12.4 ± 4**	60.1 ± 10
n-Pentyl	1.33	>200	**15.6 ± 2**	56.5 ± 10
Methoxy-ethyl	1.26	100 ± 29	**43.7 ± 8**	**186.5 ± 8**

Experimental *in vitro* evaluations of a series of alkyl esters of gentisic acid as potential skin-lightening agents using tyrosinase enzyme, melanogenesis, and cytotoxicity assays. The relative mass of each compound is denoted with respect to MG.

the series, with an IC_{50} of about 11 μg/mL. The ethyl ester of gentisate also met our criteria for an effective enzyme inhibitor. The IC_{50} of the *n*-propyl ester was 29 μg/mL. This is half the IC_{50} for the branched *i*-propyl ester, 58 μg/mL, suggesting that the active site prefers the unbranched *n*-propyl ester shape. The IC_{50} of the *n*-butyl ester was 128 μg/mL, or more than four times the value of the *n*-propyl ester, and suggests that the binding site might accommodate up to three carbon atoms beyond the ester. Methoxy-ethyl ester had an IC_{50} of 100 μg/mL. This is smaller than the value for the *n*-butyl ester, although the two compounds are essentially the same size. The *n*-pentyl ester is the largest compound that we tested in the ester series. It was not effective as a tyrosinase inhibitor ($IC_{50} > 200$ μg/mL), suggesting a size limit to that part of the active-site cavity. The differences in mass among this series had little effect on activity (i.e. when expressed in molar terms). The largest of these compounds (*n*-pentyl; 224 Da) is about one-third more massive than the smallest (methyl; 168 Da), yet its activity was over 20-fold less.

Column 4 of Table 2 displays MelAb cell pigmentation IC_{50} values for these gentisate esters. Each compound inhibited melanocyte pigmentation (both synthesis and total cell number) within the criterion we set for efficacy. Interestingly, the *n*-propyl, *n*-butyl, and *n*-pentyl esters, which are less effective tyrosinase inhibitors, were the most effective agents of the series on cultured cells, with IC_{50} values of near 15 μg/mL, albeit due to increased cytotoxicity characteristics. The next most effective depigmentation agents of the series were the methyl, ethyl, and isopropyl esters, with IC_{50} values of about 30 μg/mL against intact melanocytes. Interpretation of the activities of some of the larger esters is difficult, since many factors likely contribute to the overall "depigmentation" of melanocyte cultures (i.e. combination of inhibition of tyrosinase expression or activity and/or other components in the melanogenesis pathway, cytostasis, and cytotoxicity).

Column 5 of Table 2 presents melanocyte cytotoxicity results for the seven alkyl esters. Most of the compounds were cytotoxic, and only MG, with an IC_{50} of about 119 μg/mL, and methoxy-ethyl-ester gentisate, with an IC_{50} of about 187 μg/mL, met our arbitrary criterion for candidacy. It is interesting that the methoxy-ethyl-ester, which did not inhibit the tyrosinase enzyme, and was non-cytotoxic by our criteria, is a depigmentation agent. However, it is not likely via action on tyrosinase enzyme, and the reason for this behavior is not known.

DISCUSSION

In this work we presented a multi-endpoint screening strategy for the discovery of safe and effective active ingredients for topical skin-lightening or depigmentation products. Candidate compounds are assessed in MelAb cell cultures [6] for their ability to inhibit pigmentation of melanocytes and to determine their toxicity. Compounds that inhibit melanocyte pigmentation with low cytotoxicity are concomitantly assessed for inhibition of mammalian tyrosinase [1] using cell-free extracts of MelAb cells. If candidate compounds prove efficacious and non-toxic by these criteria, tests can be performed to determine if they are mutagenic, using a mammalian V79 cell assay, described here. Further testing using, for example, animal models can then be performed as warranted. The strategy described here is the minimum criterion for the discovery of candidate skin-lightening agents using *in vitro* methods.

We believe that candidate skin-lightening agents should satisfy at least the following criteria before further testing. First, candidate compounds ought to be enzyme inhibitors, preferably with the capacity to coordinate with the coppers in the active site of tyrosinase. This property is likely to confer some specificity to tyrosinase, since two-copper complexes are rare, and copper coordination can also increase binding and thus improve inhibition. Second, candidate compounds must exhibit low toxicity. Unfortunately, some compounds with the capacity to coordinate metals can be very toxic, and must be ruled out. Some compounds are non-toxic to non-melanocyte cells (e.g. keratinocytes), but become toxic when acted upon by tyrosinase and converted into quinones within melanocytes. HQ, for example, is more toxic to melanocytes than non-melanocytes [22], but is also generally toxic and mutagenic [6, 10, 11; this work]. Arbutin is a prodrug form of HQ [23], and is much less toxic, but does not sufficiently inhibit cell pigmentation or enzyme activity by the criteria presented here. Candidate compounds must actively or passively traverse two intact membranes at moderate concentrations without metabolic bioinactivation in order to act on living melanocytes. This requirement likely eliminates a large number of otherwise potentially effective enzymatic inhibitors. For instance, we speculate that kojic acid [24], a copper chelator with enzymatic inhibitory activity against the free enzyme, fails to enter the cells. Some compounds, such as magnesium L-ascorbyl-2-phosphate [25], are non-toxic and might work at very high concentrations, but are not effective candidates by our criteria. Lastly, visual microscopic inspection of compound effects on cell morphology is an essential part of the evaluation of candidate skin-lightening agents, as an indirect index of cytotoxicity. To our knowledge, only MG (and perhaps ethyl gentisate) satisfies all of these candidacy criteria for a topical skin-lightening agent.

Having analyzed more than 150 compounds in detail [5, 6; this work; and unpublished results], it is our general impression that testing at only a single concentration point, 40 μg/mL, is sufficient during the primary cell-based screen. Compounds that produce a lightening effect at this concentration are usually worthy of further study. Agents that render cells with normal morphology and pigment levels at this concentration are ineffective, whereas compounds that are cytotoxic at this concentration are excludable. Except for MG, which permits cell viability at this concentration, all other alkyl esters yielded cells with some evidence of toxicity.

In the present study, we demonstrated that MG can inhibit mammalian tyrosinase in pure form, in crude cell-free extract, in addition to inhibition of melanin formation in cultured melanocytes. These findings indicate that MG can enter viable melanocytes at sufficient concentrations to block tyrosinase activity and thus *de novo* melanin biosynthesis. MG does not appear to inhibit DOPAchrome tautomerase (TRP-2) or DHICA oxidase (TRP-1) at concentrations higher than needed to inhibit tyrosinase [1, 2]. A limited GA structure–activity relationship study has been performed, and only MG demonstrated the capability of blocking pigmentation in viable melanocytes at subtoxic doses [6]. These properties appear to be unique to the methyl ester of the natural product GA. Various related compounds were screened and did not have these characteristics, including GA, HQ, the 3,4-dihydroxy isomer of MG, and other simple phenolic derivatives [6; and this work]. The other compounds were either inactive or toxic in the melanocyte cell-based assays. Furthermore, measurement of the IC_{50} of GA in cell-free tyrosinase assays revealed that it was significantly less active as an enzyme inhibitor compared with MG (data not shown), and we speculate that the acid form might be less likely to enter cells than the "blocked" methyl ester derivative.

We confirmed the previously reported mutagenicity and cytotoxicity of HQ. However, the mutagenic activity was somewhat weaker than that observed in previous studies [10, 11]. We suspect that auto-oxidation products might have caused the effects. Whereas HQ was mutagenic and cytotoxic against mammalian V79 cells, no mutagenic effects were detected with either MG or GA. Perhaps the presence of the double bond of the carbonyl moiety adjacent to the benzene ring prevents auto-oxidation and conversion into the respective semiquinones and quinones (likely to be more toxic and mutagenic), whereas auto-oxidation occurs more readily with HQ. These mutagenesis results (a reconfirmation of published results) are significant, as HQ has been an approved ingredient for topical depigmentation products for many years [3]. In view of these findings (i.e. mutagenesis, cytotoxicity, and poor tyrosinase enzymatic inhibition by HQ), we speculate that HQ may have a difficult time receiving pre-market approval or continued approval during post-market safety evaluation, if it were being reviewed today by the U.S. FDA or equivalent regulatory agencies worldwide. Regulatory authorities seldom approve agents with known deficiencies, such as mutagenic potential, except for use in life-threatening or acute disease indications. Although topical HQ is moderately effective and typically does not demonstrate overt signs of toxicity *clinically*, it is certainly debatable whether topical HQ acts as a mutagen on human skin *in vivo*, and it is not desirable to intentionally expose viable tissue to known mutagens if a non-mutagenic alternative is available. This is especially true for individuals who use either high dose formulations (>4% HQ) or expose large surface areas to topical treatment. Furthermore, the National Toxicology Program has examined HQ in long-term rodent gavage studies, and has demonstrated some evidence of carcinogenic activity [26]. The demonstrated toxicity, mutagenic activity, and poor enzymatic properties of HQ in mammalian cells *in vitro* raise questions of concern about its continued use in topical products, and provide the opportunity for discovery and development of new safer replacements, of which MG serves as a candidate for further testing.

How might MG inhibit tyrosinase? Historically, Kubowitz [27] first remarked upon a strong similarity between tyrosinase and hemocyanin. Since that time a number of studies have been reported regarding a variety of tyrosinases, their mode of action, substrates, and inhibitors of the enzyme (see, for example, Yasunobu [28] for a classic review). However, the structure of mammalian tyrosinase is still unknown, so one must use indirect methods to address mechanistic issues. After noting that six histidines in tyrosinase are homologous to six histidines in hemocyanin (that coordinate two coppers in its putative active site), Lerch [29] proposed a mechanism of action for tyrosinase. Maddaluno and Faull [30] also suggested that the two coppers will chelate competitive inhibitors. Mutagenesis experiments by Spritz *et al.* [31] generally support elements of Lerch's active site model, with specific differences noted in the histidines that coordinate the coppers.

Based on the evidence gathered here, we suspect that short chain esters of gentisate bind tyrosinase by coordinating the two coppers in the active site, possibly in the presence of oxygen, as depicted in Fig. 4. We show the coppers in octahedral coordination, although the exact nature of the active-site complex remains to be determined. The ester subgroup R_2 is depicted in steric conflict with unknown residues in the active site (which we represent with an arc). This notion is supported by evidence that the branched *i*-propyl-ester gentisate is a lesser inhibitor than unbranched *n*-propyl-ester gentisate. Size limitations on the cavity for even the linear ester moieties place some constraints on R_1. This is consistent with our data that smaller linear esters of GA are better inhibitors than larger. We are currently examining in detail the biochemical mechanism of inhibition of tyrosinase by MG (Curto EV and Dooley TP, unpublished results). Furthermore, it will also be of interest to determine the extent of metabolism of MG in cultured cells or viable skin. For instance, it is not yet known whether MG is bioinactivated by esterases, sulfotransferases, or glucuronosyl transferases, for which this compound might serve as a substrate.

Our goal is to obtain additional active and safe topical agents, preferably with higher potency than MG, and then demonstrate clinical safety and efficacy in animal models. However, the dermatology/cosmeceutical field is in need of relevant and predictive mammalian *in vivo* efficacy testing models for skin-lightening/depigmentation formulations [5]. New animal models could help to verify that MG or other new compounds can be effective topical skin depigmentation agents, prior to clinical testing on human skin. Of the more than 150 compounds that we have tested thus far, MG appears to be the best candidate active ingredient.

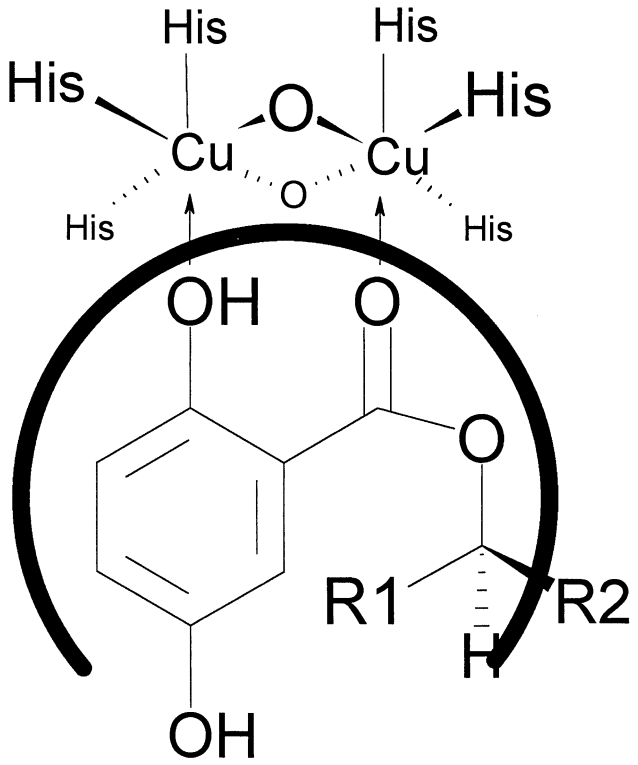

FIG. 4. Conceptual model of gentisate ester binding to the active site of tyrosinase: Hypothetical two-dimensional model of initial docking of gentisate ester in the putative active site of mammalian tyrosinase. The two coppers are assumed in octahedral coordination with a gentisate ester and oxygen.

It inhibits melanocyte pigmentation at moderate concentrations, with minimal cytotoxicity, and specifically inhibits tyrosinase enzyme. Furthermore, it is non-mutagenic, unlike HQ. No other compound that we are aware of in the literature has all of these desired characteristics, thus establishing MG as the superior candidate.

Once MG-containing topical formulations are developed and tested *in vivo* in animal models and/or clinically, what types of products could be marketed? Pre-market approval and regulatory compliance issues will have significant effects on the potential commercial availability of MG-containing products in many countries. If the product's marketing literature or labeling asserts that it alters the structure or function of skin or makes a specific "therapeutic" claim (e.g. treatment of a dermatologic disorder), this compound would likely be formulated for use as an OTC or prescription drug product. However, in selected global markets, MG-containing products could be sold as cosmetic or cosmeceutical agents [4, 5]. Cosmeceuticals represent an emerging concept in dermatologic agent development and marketing, ideologically somewhere between OTC drugs and cosmetic products, yet vaguely defined and not formally recognized by the U.S. FDA [4]. Cosmeceutical products, unlike cosmetics *per se*, should contain ingredients that have been demonstrated in the laboratory to be pharmacologically active. In addition, it is preferable if the pharmacologic action and bioavailability of the agent are limited to the viable epidermis to reduce the potential of toxicologic risks. In theory, cosmeceuticals closely resemble OTC products, with the possible exception of limiting their use only for vanity purposes (i.e. enhancement of appearance) as opposed to treatment of disease, as mandated by governmental regulatory agencies. We and others in this field are advocating for the establishment of either recommended or mandatory safety assessment guidelines for cosmeceuticals (i.e. intended for "vanity" uses only), however, without requiring demonstrations of efficacy (which are necessary for OTC or prescription drugs) [4]. Inhibitors of tyrosinase, such as MG, serve as a new paradigm in dermatologic drug or cosmeceutical agent discovery research, in view of their recently developed potential and recognition by world markets.

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