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Comparative efficacy and safety of deoxyarbutin, a new tyrosinase-inhibiting agent

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Synopsis

Several tyrosinase inhibitors have been developed and utilized to ameliorate various cutaneous hyperpigmentary disorders and complexion discolorations. Deoxyarbutin (dA) (i.e., 4-[(tetrahydro-2H-pyran-2-yl)oxy]phenol), designed using quantitative structure-activity relationships (QSAR), demonstrates effective inhibition of mushroom tyrosinase and skin-lightening capability (1). However, its comparative safety, effectiveness, and reversibility to other known tyrosinase inhibitors in human melanocytes had not been determined. The effect of dA was assessed in cultured human skin cells, on xenographs, and with a clinical trial. Using cultured human melanocytes, the maximum concentration of dA that allowed 95% viability was fourfold greater than for hydroquinone (HQ), indicating that dA is less cytotoxic/cytostatic than HQ. The viability of cultured human keratinocytes and fibroblasts was also less compromised by increasing concentrations of dA as opposed to HQ. At the maximum concentration allowing normal cellular viability, dA effectively inhibited tyrosinase activity and melanin content in human melanocytes, whereas HQ was marginally inhibitory. Upon removal of dA, tyrosinase activity and melanin content was normalized within five days. Topical application of dA on human xenografts resulted in a gradual and visually apparent skin lightening effect during an eight-week period. In a clinical trial, dA facilitated fading of pre-tanned skin to a statistically significant greater extent than either HQ or no treatment. These results demonstrate that dA is a potentially safe, effective, and reversible tyrosinase inhibitor.

INTRODUCTION

Melanin in an excessive amount and/or altered disposition is the basis for a number of congenital and acquired hyperpigmented skin diseases (2). Hyperpigmentation diseases also manifest psychosocial and cosmetic problems since they are common on sun-exposed

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areas of the face and the neck (3,4). In addition, maintaining a uniform and lighter basal skin tone is a global concern that is a result of personal preferences and/or cultural biases. Due to the therapeutics and socio-economic importance of altering skin pigmentation, many efforts have been made to develop and recognize compounds that act as depigmenting agents. Various remedies are available in the market, but none are completely satisfactory (4). Therefore, the development of an effective, controllable, and safe agent to regulate melanin synthesis in the skin is of great interest to medical practitioners and their patients.

As a result of the key role played by tyrosinase in melanin biosynthesis, most popular skin depigmenting products use a tyrosinase inhibitor as an active ingredient (e.g., hydroquinone, kojic acid, arbutin). The success of treatment using these products is limited for two basic reasons: safety or overall effectiveness. Hydroquinone (HQ) has been banned in cosmetic use in Europe and is currently available only by prescription. It is considered a melanocyte cytotoxic agent as a consequence of its oxidation, through tyrosinase and/or spontaneously, to highly reactive species such as hydroxybenzoquinone and p-benzoquinone; toxic products capable of disrupting fundamental cellular processes (4–7). Kojic acid (KA), an inhibitor of tyrosinase, has high sensitizing potential (8,9) and recently has been banned in Japan because of mutagenecity concerns (10). The effectiveness of KA has been demonstrated both *in vitro* (11,12) and in clinical trials (13,14). However, a relatively recent study demonstrated that KA treatment is not associated with reduction in pigmentation, using a cell-based assay (15). Arbutin (AR) has been traditionally used in Japan to treat pigmentary disorders (16,17), but its effectiveness is controversial. While several studies have demonstrated that arbutin can inhibit tyrosinase activity in cultured human melanocytes (17,18), many studies have demonstrated that arbutin increases pigmentation (19,20), or ineffectively inhibits tyrosinase activity both in the cell-free and cell-based assays (15).

Because of the need to provide a skin depigmenting agent that is more efficacious, more stable, and less cytotoxic than the available skin-depigmenting agents, a novel compound, deoxyarbutin (dA); 4-[(tetrahydro-2H-pyran-2-yl)oxy]phenol, was developed using quantitative structure-activity relationships (QSAR) of tyrosinase inhibitors (1). In this report, we analyze the safety, effectiveness, and reversibility of dA on human melanocytes in comparison to the aforementioned tyrosinase inhibitors, hydroquinone, kojic acid, and arbutin. In addition, we demonstrate the skin lightening effect of dA on human skin.

MATERIALS AND METHODS

CHEMICALS AND SOLVENTS

Hydroquinone (HQ), kojic acid (KA), arbutin (AR), and 4-tertiary butylphenol (TBP) were purchased from Sigma Chemical (St. Louis, MO). Deoxyarbutin (dA) was initially synthesized by the authors and scaled up by Girindus America Inc. (Cincinnati, OH). For culture studies, dA, HQ, KA, and TBP were dissolved in sterile DMSO, while AR was dissolved in MCDB153 media (Irvine Scientific, Santa Ana, CA). TBP was included in this comparative study because it is a tyrosinase inhibitor implicated in contact/occupational vitiligo (21). Stock solutions of compounds were prepared and protected

from light at −20°C until use. At the time of use, the compounds were further diluted in growth medium to the final concentration. For the animal study, 5% dA, HQ and TBP were prepared in a mixture of propylene glycol, ethanol, and water at a volume ratio of 1:2:1. Solutions were kept at −20°C until use.

CELL CULTURE

Primary cultures of normal human melanocytes, keratinocytes, and fibroblasts were established from individual neonatal foreskins [from dark (dk) and light (lt) skin infants] that were obtained from the nursery of the University Hospital in Cincinnati after routine circumcision using a protocol approved by the University of Cincinnati Institutional Review Board as previously described (22). Foreskins were incubated in 0.25% trypsin for two hours at 37°C. The tissue was gently vortexed for 30 s to separate the dermis as a single piece and produce an epidermal cell suspension. The epidermal cells were seeded in a T-25 cm^2 flask in either melanocyte or keratinocyte growth medium. Melanocytes were maintained in MCDB-153 growth media (Irvine Scientific, Santa Ana, CA) supplemented with 4% fetal bovine serum, 1% antibiotic/anti-mycotic solution (Gibco, BRL, Grand Island, NY), 1 µg/ml vitamin E, 0.6 ng/ml human recombinant basic fibroblast growth factor, 5 µg/ml insulin, 0.05 µg/ml transferrin, 13 ng/ml bovine pituitary extract (Clonetics, Walkersville, MD) and 8 nM 12-O-tetradecanoylphorbol-13-acetate. All of the above reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. The growth medium for normal human keratinocyte cultures consisted of M154 basal medium (Cascades Biologicals, Portland, OR) supplemented with human keratinocyte growth supplement (Cascade Biologicals) and 1% antibiotic/anti-mycotic (Gibco).

The dermis was vortexed and seeded into a T-25 cm^2 flask with fibroblast growth medium consisting of DMEM medium (Gibco) containing 8% fetal bovine serum, 1% glutamine (Gibco), 1% sodium pyruvate (Gibco) and 1% antibiotic/anti-mycotic solution (Gibco).

All cultures were maintained in a tissue culture incubator at 37°C with 5% CO_2. The growth medium was routinely changed twice a week for melanocytes and fibroblasts and every other day for keratinocytes.

CELL VIABILITY ASSAY

Cell number (i.e., viability) was determined by direct counting. In this technique, cells were seeded at 1.3×10^5 cells for melanocytes, 5×10^4 cells for fibroblasts, and 5.5×10^4 cells for keratinocytes, per T-12.5 cm^2 flask. Cells were allowed to attach and grow for 48 hr before treatment. Cells were then treated daily with fresh growth media containing test compounds for five days. Compounds were tested at a range of concentrations in order to determine the maximum dose that did not affect the viability of human cells (experiments were performed at least in duplicate). On the sixth day, cells were detached with 1X trypsin/EDTA and counted with a Coulter Counter. To determine the fraction of cells surviving the treatment in a specified concentration of an experimental agent, the viable cell number after each treatment was normalized using the average of the viable cell number in a control group.

IN SITU (INTACT) TYROSINE HYDROXYLASE ASSAY

Established melanocytes from light and dark skin were subcultivated in six-well plates at a density of 1×10^5 cells/well. Cells were treated daily in triplicate for five days with fresh growth media containing different dosages of test compounds. On the fifth day cultures were assayed for tyrosine hydroxylase activity as previously described (22,23). In short, cells were fed with fresh media containing 1 μCi/ml of H^3-tyrosine (Amersham Pharmacia Biotech, Piscataway, NJ) and the test compound. After 24-hour incubation, media from each well were collected and diluted in an equal volume of 10% (w/v) activated charcoal in a 0.1 N citric acid solution. Duplicate 1-ml aliquots of the charcoal/media mixture were passed through a Dowex® 50W×8-200 acidic cation 1.0-ml exchange column (Sigma-Aldrich, St. Louis, MO) followed by a 1-ml 0.1 N citric acid solution wash. The radioactivity of the tritiated water in the eluate was counted in a Packard 1900 CA liquid scintillation analyzer (Packard Instrument Company, Meriden, CT). The cultured cells in each well were harvested by trypsinization, and the cell suspension was used to determine protein and melanin content. The tyrosinase activity (DPM/24 hours/μg protein) of the cells after treatment was normalized to the activity of control cells.

MELANIN AND PROTEIN CONTENT ASSAY

Melanin content was determined as previously described (22). In short, cultured cells in each well were harvested with 0.2% trypsin. Cell suspensions were washed twice in phosphate-buffered saline (PBS). The cell pellets were dissolved with 100 μl Triton-X-100 and lysates centrifuged at 13000 rpm at 4°C for 20 min. The supernatants were used to determine protein content, and pellets were used to determine melanin content. The pellets were washed with 50 μl of ethanol:ether (1:1), lysed in 100 μl of 0.2 N NaOH in 20% DMSO, and the absorbance was measured at 450 nm using a microplate reader (Bio-Rad Model 550, Japan). The melanin content of cells after treatment was expressed as μg melanin/mg protein after normalization with the control.

Protein content was determined using the BCA assay (Pierce Chemical, Rockford, IL). In brief, 10 μl of supernatant was added to 200 μl of substrate (50 parts reagent A/1 part reagent B) in a 96-well plate. The plate was incubated for 30 minutes at 37°C and then the absorbance was measured at 570 nm in a microplate reader (Bio-Rad Model 550, Japan). The absorbance was compared with a standard curve established using known concentrations of BSA (Pierce Chemical).

REVERSIBILITY ASSAY

To determine if the effect of dA is reversible, inhibition of tyrosinase activity and melanin synthesis was assayed in a pulse-chase manner. Established melanocytes from dark skin donors were subcultivated in six-well plates at a density of 1×10^5 cells/well. Experiments were done in triplicate using a previously determined optimal effective dosage of dA that allowed 95% viability. After five days, a group of dA-treated and vehicle cells were assayed for the inhibition of tyrosinase activity and reduction of melanin content to demonstrate the effect of the test compound. Simultaneously, in another dA-treated group, treatment was halted and this group was fed daily with fresh

growth media for an additional five days. At the same time, one of the vehicle-treated group had vehicle treatment halted and this group was fed daily for another five days with fresh growth media. On day 11, the four remaining groups (vehicle-treated for ten days, dA-treated for ten days, dA-treated for five days and then untreated for five days, and vehicle-treated for five days and then untreated for five days) were assessed for tyrosinase activity and melanin content. The same experimental procedure was performed for HQ and TBP.

XENOGRAFTING

Xenografts were developed using a protocol approved by the Cincinnati Children's Hospital Medical Center IACUC with animal welfare assurance. Female ICR-SCID mice (Taconic, NY) kept under pathogen-free conditions (Cincinnati Children's Hospital Research Foundation, Cincinnati, OH) were shaved with an electric clipper to remove the dorsal hair. The mice were anesthetized by isofluorane/oxygen (3%/0.8 liter). The dorsal site was cut to produce a wound bed of approximately 2.0–3.0 cm in diameter. Fresh split-thickness cadaveric skin (U.S. Tissues and Cells, Cincinnati, OH) from a Caucasian donor was sutured in place with a reversed cutting precision monofilament PS-3, 6-0 (Moore Medical, CT). Grafts were left untreated for two months, during which time hyperpigmentation occurred. The degree of hyperpigmentation was assessed weekly using a microdigital image obtained from the Charm View™ (Moritex, Japan) surface optical imaging system. The treatment phase was initiated when no further increase in pigmentation was observed. Animals were balanced into three mice per group according to their *L* values among four treatment groups [deoxyarbutin (dA), hydroquinone (HQ), 4-tertry butyl phenol (TBP), and the vehicle-treated control group].

Treatments were topically applied at a 5% (w/v) concentration in propylene glycol, ethanol, and water at a volume ration of 1:2:1, at 12.5 μl/2 cm^2, five days per week, for eight weeks. The treatment sites were assessed on a biweekly basis for the degree of pigmentation using the Charm View™ system. This photographic system took enlarged digital images of the treatment sites, and then with Universal Serial Bus (USB) capture equipment, the images were transferred to a computer. Subsequently, the color parameters for these images (*L, a, b*) were obtained by using Adobe® Photoshop® software (Adobe Systems Inc., San Jose, Calif.).

IMAGE ANALYSIS

By using a histogram function in Adobe® Photoshop® software (Adobe Systems Inc.), the color or tonal range of the digital image can be evaluated in either RGB or *L, a, b* mode. This software allows the desired region of the image to be selected and analyzed. This includes mean, standard deviation, median, and the number of pixels of each color parameter.

The *L* a* b* system is an international standard system, recommended by the CIE (Commission Internationale de l'Eclairage) in 1976 for skin color assessment. In this system, *L* a* b* color consists of a lightness component (*L**), which ranges from 0 to 100, and two chromatic components: *a** (from green to red) and *b** (from blue to yellow).

Both a^* and b^* range from −120 to +120 (Adobe® Photoshop® 7.0 User Guide for Windows® and Macintosh).

Adobe® Photoshop® uses values of 0 to 255 to characterize L, a, and b values of the selected image. L is the luminance. It gives the relative brightness from total black (L=0) to total white (L=255). The a value represents the balance between green (a=0) and red (a=255). The b value represents the balance between blue (b=0) and yellow (b=255). In this report, the L value was used to assess the lightening effect of the applied treatment and the a value was used to assess the occurrence of irritation (redness) as a result of the treatment. The L and a values obtained from Adobe® Photoshop® were converted to L^* and a^* values of the CIE color model by using the following formulas (24):

$$L^* = (L/255)\,(100) \tag{1}$$

$$a^* = (240a/255) - 120 \tag{2}$$

HISTOLOGY AND IMAGE ANALYSIS

Biopsies at the eight-week time point were processed for histology and stained with hematoxylin and eosin (H&E) and Fontana-Mason with nuclear fast red counterstain (F&M) by the Dermatopathology Laboratory in the Department of Dermatology at the University of Cincinnati. Three sections per biopsy and five different areas for each section were analyzed as follows: Images of the H&E and F&M sections were captured by a Spot Insight 4 megapixel-digital camera and Spot Imaging Software version 3.2 (Diagnostic Instruments, Inc., Burroughs, Sterling Heights, MI) attached to a light microscope using the 20 × objective. The H&E sections were analyzed for the presence of inflammation or aberrant morphology. The F&M sections were used to assess the percent of melanin per epidermal area. Fifteen images from tissues sections of each group were analyzed. For each image, the stratum corneum and the dermis were manually extracted using Adobe® Photoshop® version 7.0. Then each image in .tif format was loaded in the MATLAB® program, version 6, in RGB mode. The algorithms written in MATLAB® evaluated the red color along the (X,Y) coordinators, and saved these (X,Y) coordinators for later use in calculating epidermal size. The amount of melanin in the tissue was subsequently assessed from a duplicate image in which the original image was converted to a gray scale. Prior to a thresholding process, the red pixels that corresponded to the saved (X,Y) coordinators were removed. This was accomplished by changing these (X,Y) coordinators to white (R, G, B = 255). The total number of black pixels corresponded to the amount of silver-stained melanin, whereas the sum of the black and red pixels represented the size of the epidermis. Finally, percent melanin in the epidermis was calculated by dividing melanin by the epidermal size and multiplying by 100.

CLINICAL TRIAL

A human clinical trial was performed over a six-week period with 25 male and female subjects, ages 18–60, with Fitzpatrick skin types of III or IV. Three skin sites on the back of each subject were exposed for 10–20 minutes daily for seven consecutive days to UV light from a tanning bed (Cosmolux AS bulbs emitting 2.6% UVB at 260–320 nm and 97.4% UVA at 320–400 nm). At the end of the tanning regimen, one of each of the

three coded test sites was (a) left untreated, (b) treated with 3% dA, or (c) treated with 4% HQ. Treatments were topically applied three times per week for five weeks, at 12.5 μl/2 cm^2 in moisturizer (oil-in-water emulsion type), using an occlusive patch system, modified to a semi-occlusive system when necessary to manage irritation. For skin lightness evaluation, L values were obtained using the Minolta Chroma Meter at the end of tanning (baseline) and termination of study (five weeks), and the percentage of tan retained was quantitated (i.e., L value at end of trial/L value at beginning of treatment × 100) at the end of the trial. Written informed consent compliant with 21 CFR 50.25 was obtained from each subject prior to enrollment in the study. Subjects and graders were blinded to the identity of each product.

STATISTICAL ANALYSIS

Data from the cell culture and the clinical trial were subjected to statistical analysis using one-way analysis of variance (ANOVA) in conjunction with Student's t-test. One-way ANOVA was used to determine if there were differences among groups, and then multiple comparison procedures (Bonferroni t-test) were used to isolate these differences. Differences were considered significant if $p < 0.05$.

Animal results (ΔL and Δa) were analyzed as a one-between (subject) one-within (time) repeated-measurement analysis of variance using the PROC MIXED model in SAS. The Bonferroni correction of the p-value was calculated by dividing the test-wise p-value by the number of comparison groups (p-value for our animal study = 0.004). For epidermis size results, One-way ANOVA and the Bonferroni t-test were used to compare treatment groups with the vehicle control group. Differences were considered significant if $p < 0.05$.

RESULTS

COMPARATIVE EFFECT OF TYROSINASE INHIBITORS ON THE VIABILITY OF CULTURED HUMAN MELANOCYTES, KERATINOCYTES, AND FIBROBLASTS

The effect of each tyrosinase inhibitor (i.e., dA, HQ, KA, AR, and TBP) on the viability of human melanocytes derived from both light (lt) and dark (dk) skin was evaluated in a dose-dependent manner (Tables I and II). The maximum concentration of dA that allowed 95% viability (Table II) was fourfold higher than that of HQ on both dark and light human melanocytes. In addition, light melanocytes could tolerate a fourfold higher concentration of either dA or HQ than dark melanocytes. In contrast, KA, AR, and TBP all exhibited a maximum concentration of 50 μM for both dark and light melanocytes.

The effect of each tyrosinase inhibitor on the viability of human fibroblasts and keratinocytes was evaluated in a dose-dependent manner (Figures 1 and 2). HQ exhibited a statistically significant inhibition of viability in human fibroblasts and keratinocytes compared to dA at 25 μM, 12.5 μM, and 6.25μM (Figure 1). At the maximum concentration tested (i.e., 25 μM), loss of viability in keratinocytes due to HQ was associated with cell fragmentation and detachment. This indication of apoptosis was not apparent in keratinocytes treated with dA at the same concentration at which the viability of keratinocytes was compromised (Figure 1c–e). AR and KA demonstrated less effect on

Table I
The Mean Values for Light and Dark Human Melanocyte Viability at Different dA and HQ Concentrations [The concentration that allows 95% viability (bold) was chosen for each test compound (see Table II)]

Treatment (μM)	Mean viable cell per control for light human melanocytes ± SD*	Mean viable cell per control for dark human melanocytes ± SD*
dA 25	0.795 ± 0.012**	
dA 12.5	0.877 ± 0.063	0.761 ± 0.076
dA 6.25	**0.963 ± 0.018**	0.854 ± 0.045
dA 3.125	0.986 ± 0.011	0.912 ± 0.031
dA 1.5625	1.002 ± 0.024	**0.949 ± 0.049**
dA 0.78	0.990 ± 0.021	0.971 ± 0.027
HQ 25	0.851 ± 0.023	
HQ 12.5	0.854 ± 0.025	0.814 ± 0.019
HQ 6.25	0.909 ± 0.010	0.859 ± 0.035
HQ 3.125	0.899 ± 0.035	0.910 ± 0.032
HQ 1.5625	**0.953 ± 0.047**	0.929 ± 0.006
HQ 0.78	0.969 ± 0.019	0.932 ± 0.023
HQ 0.391	0.975 ± 0.043	**0.958 ± 0.034**
HQ 0.195	0.983 ± 0.036	0.979 ± 0.024
Control	1.01 ± 0.02	1.00 ± 0.00

* Values for light and dark human melanocytes represent the mean of cell lines developed from four and six separate foreskins, respectively.
** In each individual experiment, duplicate samples were measured for each duplicate flask/concentration.

Table II
Concentrations of Tyrosinase Inhibitors That Allow for 95% Viability per Control in Normal Human Melanocytes Derived from Dark (NHM-dk) and Light (NHM-lt) Skinned Persons

Compounds	NHM-dk (μM)	NHM-lt (μM)
HQ	0.391	1.5625
dA	1.5625	6.25
TBP	50	50
KA	50	50
AR	50	50

the cell viability of keratinocytes and fibroblasts as compared to dA and HQ (Figure 2). In contrast, TBP demonstrated a significant reduction of viability in keratinocytes and fibroblasts compared to KA and AR (Figure 2).

COMPARATIVE EFFECT OF TYROSINASE INHIBITORS ON TYROSINASE ACTIVITY AND MELANIN SYNTHESIS OF CULTURED HUMAN MELANOCYTES

At concentrations that did not significantly effect viability (Table II), the efficacy of dA in inhibiting tyrosinase activity and reducing melanin content in dark human melanocytes was determined and compared with the efficacy of HQ, KA, AR, and TBP. Within intact dark human melanocytes, dA significantly inhibited tyrosinase activity with a concomitant significant reduction in melanin content (Figure 3a,b). KA and TBP also significantly inhibited tyrosinase activity and melanin content. In contrast, HQ exhibited a significant reduction in tyrosinase activity without a significant reduction in

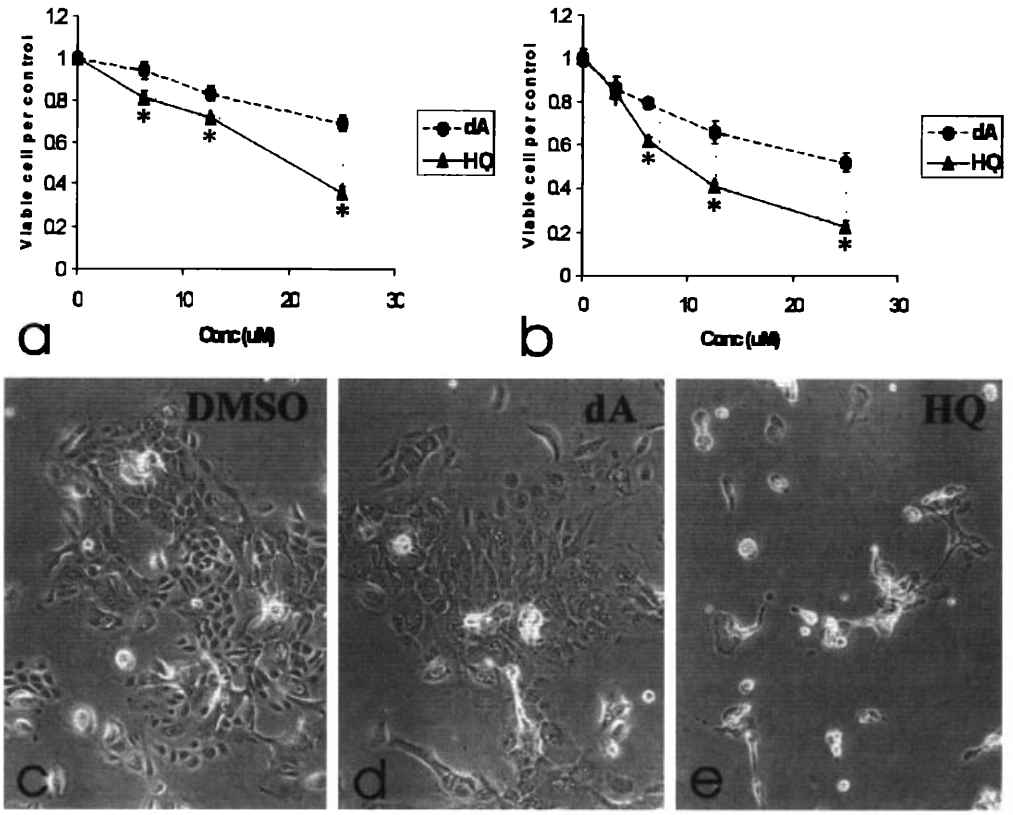

Figure 1. Viability and morphology of normal human fibroblasts and keratinocytes were less significantly affected by dA compared to HQ. Human fibroblasts and keratinocytes cultures were treated daily with fresh media containing either dA or HQ in a dose-dependent manner for five days. On day 6, cells were detached and counted with a Coulter Counter. The cell number of the treated flask was divided by the mean cell number of the vehicle-control flasks and plotted vs the test compound concentrations. (a) Effect of dA and HQ on viability of normal human fibroblasts. Duplicate samples were measured for each duplicate flask/concentration. (b) Effect of dA and HQ on viability of normal human keratinocytes. Results are expressed as the mean of triplicate cultures/concentration. (c–e) Morphology of keratinocytes cultures treated with (c) vehicle alone, or with 25 μM of (d) dA, or (e) HQ. * = Significant difference between the dA- and HQ-treated groups at the same concentration at $p < 0.05$.

melanin content (Figure 3a,b). AR did not alter either tyrosinase activity or melanin content. Within intact light human melanocytes, dA, KA, and TBP significantly inhibited tyrosinase activity (Figure 3c). In contrast, HQ exhibited no significant inhibition of tyrosinase activity in melanocytes from light skin (Figure 3c). AR-treated melanocytes exhibited enhanced *in situ* tyrosinase activity as compared to the untreated control.

REVERSIBILITY OF TYROSINASE INHIBITORS IN CULTURED HUMAN MELANOCYTES

The time-dependent inhibition and the reversibility of inhibition on the tyrosinase activity and melanin content of dark human melanocytes treated with dA was assessed and compared with the effect of HQ and TBP at concentrations that did not significantly affect viability. Treatment of dark melanocytes with dA and TBP for five and ten days

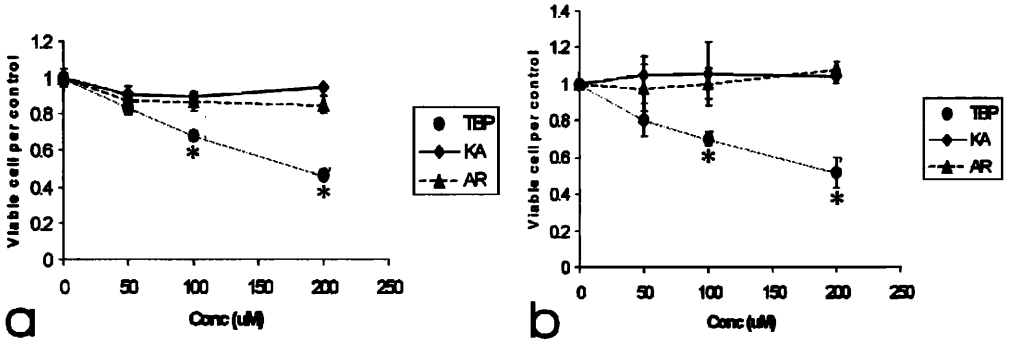

Figure 2. Viability of normal human fibroblasts and keratinocytes was unaffected by AR and KA and significantly affected by TBP. Human fibroblast and keratinocyte cultures were treated daily with fresh media containing AR, KA, or TBP in a dose-dependent manner for five days. On the sixth day, cells were detached and counted with a Coulter Counter. The cell number of the treated flask was divided by the mean cell number of the vehicle-control flasks and plotted v. the test compound concentrations. (a) Effect of AR and KA on viability of normal human fibroblasts as compared to the effect of TBP. Duplicate samples were measured for each duplicate flask/concentration. (b) Effect of AR and KA on viability of normal human keratinocytes as compared to the effect of TBP. Results are expressed as the mean of triplicate cultures/concentration. * = Significant difference from both KA- and AR-treated groups at the same concentration at $p < 0.05$.

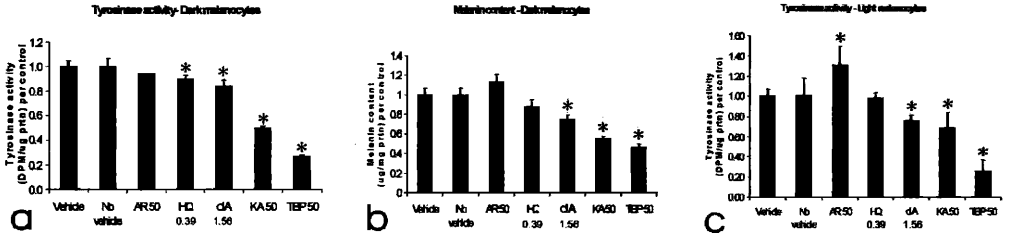

Figure 3. At a concentration that did not affect viability, dA significantly inhibited *in situ* tyrosinase activity of cultured dark and light human melanocytes, with concomitant significant reduction in melanin content. Triplicate cultures at a density of 1×10^5 cells/well of dark and light skin human melanocytes were treated daily for five days with fresh media containing the previously determined concentrations that did not significantly affect viability of each test compound. On the fifth day, cells were fed with fresh media containing 1μCi/ml of H^3-tyrosine and the appropriate compound. After 24 hours, the radioactivity of the tritiated water was counted in a Packard 1900 CA liquid scintillation analyzer, and the melanin and protein content were determined for each culture as previously mentioned in Materials and Methods. Tyrosinase activity for cells in a specified concentration of test compound was expressed as DPM/24 hours/μg protein divided by the mean DPM/24 hours/μg protein of the vehicle-control flasks. (a,b) The effect of five days treatment with dA, HQ, AR, KA, and TBP on *in situ* tyrosinase activity and melanin content of dark human melanocytes, respectively. (c) *In situ* tyrosinase activity of light human melanocytes. Results are expressed as the mean of triplicate cultures ± SD. * = Significant difference from the control group at $p < 0.05$.

significantly reduced tyrosinase activity (Figure 4a) and melanin content (Figure 4b). In contrast, HQ was similar, except that the melanin content was not significantly reduced at day 5 (Figure 4b). After halting treatment, the dA-treated melanocytes returned to statistically normal values of tyrosinase activity, whereas HQ- and TBP-treated melanocytes remained statistically reduced from the control group. Treatment with dA, HQ, or TBP resulted in a concomitant reduction in melanin content. The reduction in melanin was detected by five days of treatment for both dA and TBP and by ten days of

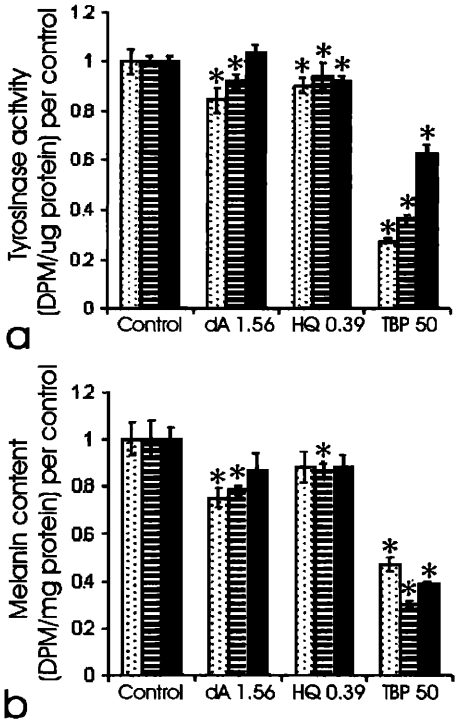

Figure 4. dA is a reversible inhibitor in which significant recovery of tyrosinase activity and melanin content occurred five days after halting treatment. Dark human melanocytes, in six triplicate groups at a density of 1×10^5 cells/well, were treated daily with fresh growth media containing each test compound at the concentration that did not affect viability. After five days, one treated group and one control group were assessed for inhibition of tyrosinase activity and melanin content reduction. At day 5, one of the treated groups had treatment halted and was fed, along with one of the control groups, fresh media for another five days. On day 11, the four remaining groups (test compound treated for ten days, vehicle treated for ten days, test compound treated for five days and then untreated for five days, and vehicle treated for five days and then untreated for five days) were assessed for tyrosinase and melanin content as described in Materials and Methods. (a) Tyrosinase activity, expressed as DPM/24 hours/μg protein divided by the mean DPM/24 hours/μg protein of the vehicle-control flask, and (b) melanin content, expressed as μg melanin/mg protein divided by the mean μg melanin/mg protein of the vehicle-control flasks, for cells treated with test compound for five days (stippled bars), ten days (striped bars), or for five days and untreated for five subsequent days (solid bars). * = Significant difference from the control group at $p < 0.05$.

treatment for HQ. Only the inhibitory effect of dA on melanin synthesis was shown to be reversible.

ANIMAL STUDY RESULTS

The effectiveness of dA on reducing hyperpigmentation compared to HQ in a xenograft model with human skin grafted onto SCID mice was assessed and compared with the effect of HQ and TBP. Three mice per group were treated five days per week for eight weeks with dA, HQ, and TBP at 5% concentration. Photos for each graft were taken every two weeks using the Charm View™ (Moritex, Japan) system and then assessed using Photoshop software for *L* (lightening) and *a* (redness) values. *L* and *a* values were

then converted to the L^* and a^* values of the CIE color model, using equations (1) and (2) presented in the Materials and Methods section.

Topical treatments for eight weeks with dA (Figure 5a) resulted in a gradual improvement in skin lightening compared to the vehicle-treated control (i.e., increase in ΔL^* values; higher values for ΔL^* indicate the occurrence of lightening). HQ and TBP treatment also resulted in a gradual increase in skin lightening. Statistical analysis demonstrated that skin lightening at weeks 4, 6, and 8 in all treated groups was statistically different from the initial L value in each group ($p < 0.0001$). The data in Figure 5a is also presented in a bar graph (Figure 5b) that includes the standard deviation (standard error, SE) per group. Standard deviation was relatively greater in the HQ- and TBP-treated groups at weeks 4, 6, and 8 than in the respective dA-treated groups. Histological sections of xenografts were obtained at the termination of treatment (i.e., week 8) and evaluated for percent melanin per epidermal area as described in Material and Methods. Treatment with all tyrosinase inhibitors resulted in a significant

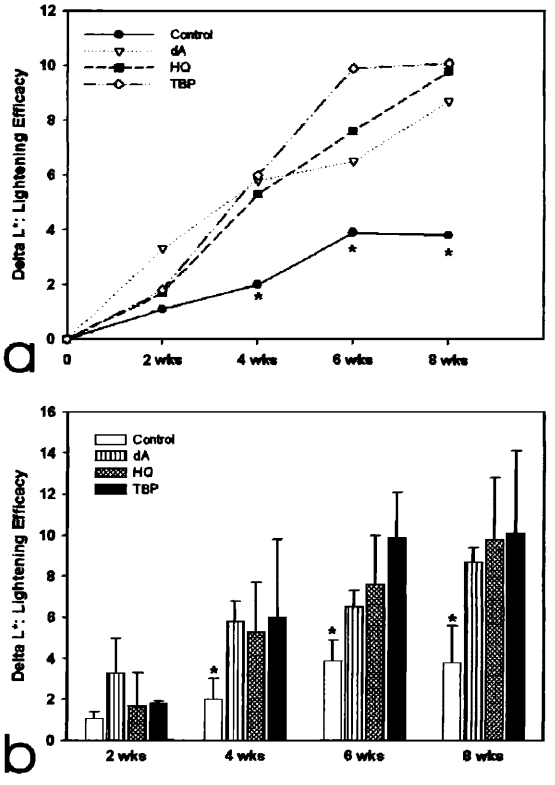

Figure 5. Topical treatment for eight weeks with dA resulted in a gradual increase in skin lightening compared to the vehicle-treated group. Human skin grafted onto mice (three mice per group) was treated with 5% test compounds, five days per week, for eight weeks. Treatment sites were assessed biweekly for the degree of pigmentation using the Charm View™ system. The L and a values for each treatment site were obtained by using Adobe Photoshop software and then were converted to the L^* and a^* values of the CIE color mode. The time course of change in L^* values from baseline value for grafts treated with dA, HQ, TBP, and vehicle are presented as (a) a line graph and (b) a bar graph depicting the mean ± SE. * = Significant difference at $p < 0.0001$ between the baseline value for each group and their values at four-, six-, and eight-week points.

inhibition of the melanin present in the epidermis (Table III). The lightening of grafting-induced hyperpigmentation was visibly apparent (Figure 6a–f). In addition, two of three mice treated with HQ developed brown coloration of the hair around the grafts not observed in mice treated with the vehicle or dA (Figure 6g-i). H&E stained sections for grafts treated with dA and HQ for eight weeks demonstrated no signs of inflammation or abnormal morphology (data not shown).

CLINICAL TRIAL RESULTS

The effect of dA and HQ on the lightening of tanned skin was compared in a clinical trial as described in the Materials and Method section. After a seven-day regimen of tanning, areas of skin were left untreated or treated blindly with either dA or HQ, three times per week for five weeks. At the end of this period, the percent of tan remaining in the untreated site was 44.6%. In comparison, the percent of tan remaining in the sites treated with either dA or HQ was 37.3% (a significant increase in tanning loss over control) versus 51.6% (a significant decrease in tanning loss over control), respectively. These results suggest that dA accelerated the fading of UV-induced tan, whereas HQ impeded this response.

DISCUSSION

Toxicity of phenolic compounds can arise from three possible mechanisms: (a) susceptibility of agents to extracellular auto-oxidation, (b) cellular permeability, and (c) intracellular oxidation by tyrosinase and/or other metabolic enzymes (25–27). Deoxyarbutin (dA), was shown to reversibly reduce tyrosinase activity and melanin content and demonstrated less cytotoxicity, as compared to HQ, in the three normal human skin cell types (i.e., melanocytes, keratinocytes, and fibroblasts).

Studies have previously demonstrated that a major component in the toxicity of phenolic compounds is attributable to reactive oxygen species produced outside the cells (25). Specifically, hydroquinone auto-oxidation was shown to occur predominantly in the extracellular environment and to be a causative event for quinone-induced cytotoxicity (27). Thus, the lower cytotoxicity of dA compared to HQ may be due to its enhanced stability and reduced auto-oxidation. The enhanced stability could be attributed to the presence of an acetyl bond in its structure that allows stability in basic conditions. In addition, and in contrast to HQ, the OH group in the *para* position forming the acetyl bond in dA would result in conferring dA less susceptible to auto-oxidation.

Table III
Percent Melanin per Epidermal Area in Xenografts Treated for Eight Weeks with Vehicle or Various Tyrosinase Inhibitors

	% Melanin/epidermal area
Vehicle	0.088 ± 0.07
dA	0.061 ± 0.06
HQ	0.043 ± 0.05
TBP	0.015 ± 0.02

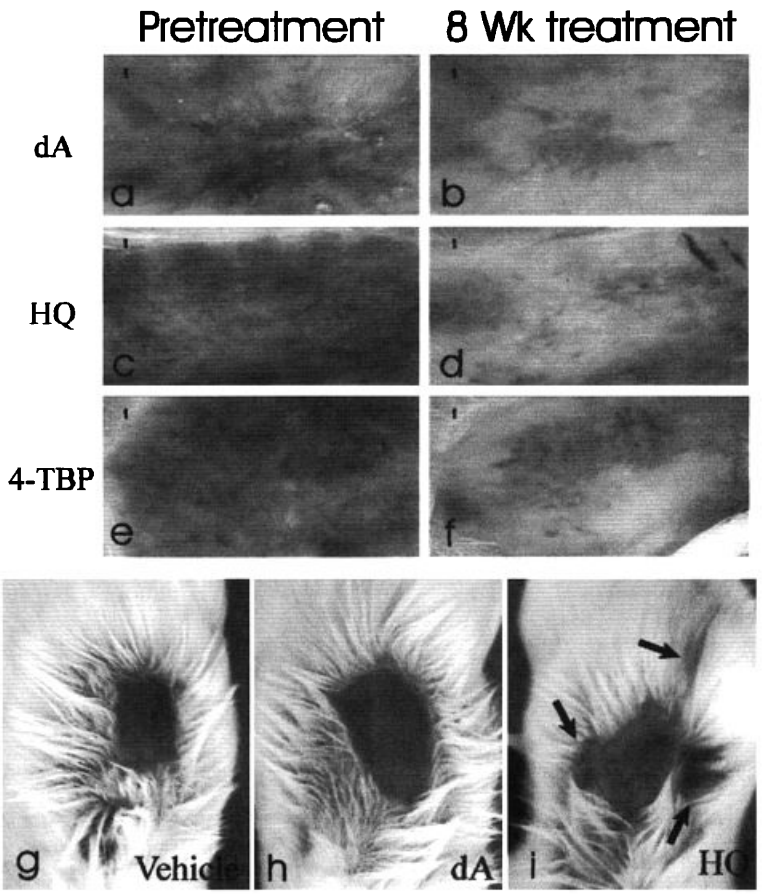

Figure 6. Grafting-induced hyperpigmentation is reduced by dA. Grafts described in Figure 5, at the onset of the treatment (a,c,e) and after eight weeks of treatment (b,d,f) with dA (a,b), HQ (c,d), or TBP (e,f), demonstrate the skin-lightening effect of each tyrosinase inhibitor. In addition, grafts treated for eight weeks with vehicle only (g) as opposed to dA (h) or HQ (i) exhibit darker versus lighter pigmentation, respectively. Note that in two of the three HQ-treated grafts a brown stain coloration adjacent to murine hair (arrows in i) is observed.

The relative cellular permeability of phenols may also correlate with the effect of the compound on cellular viability, as well as its efficacy as its tyrosinase inhibitor (25). Relatively non-polar phenols, like dA and HQ, are more permeable and thus have the potential to be more toxic as compared to more polar phenols, like AR, that less readily enter the cell (25). Our present analysis demonstrates that AR is a relatively safe compound compared to dA and HQ. However, AR is either an ineffective tyrosinase inhibitor or promotes tyrosinase activity in light-versus-dark human melanocytes, respectively. The ineffectiveness of AR as a tyrosinase inhibitory agent is in agreement with the findings of Curto *et al.* (15). However, an increase in pigmentation by AR has been previously reported in several studies (19,20). In contrast, several studies have demonstrated that AR is a tyrosinase inhibitor (17–19). However, these latter studies utilized high concentrations of AR in a culture system (17,19) and/or assessed tyrosinase inhibition using cell lysate rather than intact cells (17–19). This suggests that AR, although an effective inhibitor of the catalytic activity of isolated tyrosinase, is relatively

ineffective on intact melanocytes. The sugar residues in AR confer a significant shift in both size and polarity, e.g., the clog P of arbutin is −0.58 as compared to +0.56 for HQ, thus likely preventing it from passing through cellular membranes in sufficient concentration to inhibit tyrosinase within melanosomes. It is noteworthy to mention that, so far, the effective topical concentration of arbutin has not been formally evaluated and published (28).

Once internalized, phenolic derivatives can act as substrates for tyrosinase. This requires electron donor groups (i.e., alkoxyl and hydroxyl groups) in the *para* position with respect to the OH group (5). HQ and its esters possess an electron donor group in that position. It has been demonstrated that HQ, an effective tyrosinase inhibitor (29), can be oxidized by tyrosinase to hydroxybenzoquinone and p-benzoquinone, thus generating reactive oxygen species (6,7,30). With dA and AR, the electron-donating ability of the *para* oxygen is reduced by the presence of a second oxygen ring, thus slowing or stopping the oxidation rate. Oxidation products can be produced from arbutin by mushroom tyrosinase (31). Whether dA can also be converted to quinones and generate reactive oxygen species, and at what rate, has yet to be determined.

All tested tyrosinase inhibitors eventually demonstrated toxicity against keratinocytes and fibroblasts. This suggests that toxicity can arise from a non-tyrosinase-mediated mechanism. A peroxidase-mediated mechanism in the cytotoxic effects of melanogenic inhibitory agents, specifically phenolic compounds, has been demonstrated previously (32). Phenolic compounds can also serve as substrate and inhibitors for peroxidase (32–34). For example, HQ serves as a good substrate for peroxidase (35). Melanocytes, fibroblasts, and keratinocytes are all equipped with peroxidases as part of their antioxidant enzyme system that helps the cell to counteract oxidative stress (36). A peroxidase-mediated mechanism can explain the non-specific cytotoxicity of the tyrosinase inhibitory agents we have tested.

Putative depigmenting agents should subsequently lead to removal of unwanted pigment when applied topically to skin. Thus, the effectiveness of deoxyarbutin as a treatment to reverse hyperpigmentation of human skin grafted to immunocompromised mice was assessed and compared with hydroquinone and 4-tertiary butylphenol (TBP). Xenografting of human skin onto immunocompromised mice is an excellent model for studying various aspects of skin physiology including hyperpigmentation of grafted skin on burn patients (37). In addition, this model is more physiologically relevant in that it allows the evaluation of melanogenic inhibitors directly on human skin. Hyperpigmentation is common sequelae of most injuries to the skin (37). Farooqui *et al.* (37) showed that hyperpigmentation of human cutaneous xenografts placed on athymic nude mice was apparent as early as 4–6 weeks post-grafting. In addition, tyrosinase, a key enzyme in melanin biosynthesis, was upregulated in the grafted skin from 2 to 12 weeks post-grafting (37). Thus, topical application of a tyrosinase inhibitory agent could be an approach to reverse hyperpigmentation of the grafted skin and thus test its effect as a general skin-lightening modality. Topical application of dA reversed the skin hyperpigmentation of human skin grafted onto immunocompromised mice. Initial statistical analysis showed that treatments are statistically different within a week ($p < 0.0001$). By statistically comparing the mean values for ΔL^* to the control group values, only one treatment group (HQ) at one time point (eight weeks) was statistically significant from the control group. Although skin lightening for dA, TBP, and even HQ at the other time points was not statistically significant from that of the control group, lightening

of the grafts was obvious both visually and from the gradual positive increase in ΔL^* compared to the vehicle-treated control. The increase in ΔL^* started for dA at the two-week treatment point and increased gradually over the eight-week treatment period. In addition, TBP-treated grafts developed clear vitiligo-like patches. This discrepancy may result from the high SE and low number of animals in each group. For instance, in order to be able to detect a significant difference for the dA-treated group eight weeks after treatment, using the two-sided two-sample test, we needed at least five animals in each group.

The effect of dA on reversing hyperpigmentation demonstrated in the xenograft model system was recapitulated in a clinical trial. Hyperpigmentation induced by UV exposure was effectively reduced by dA. The lightening effect of dA on pathologic hyperpigmentary disorders like melasma, solar lentigo, and post-inflammatory hyperpigmentation is yet to be determined.

An interesting observation is that mice treated with HQ developed brown coloration of the albinistic hair adjacent to the grafts. Presumably this is a staining artifact from the by-products of hydroquinone oxidation. This brown pigmentation matches the reported nail plate discoloration that can develop during use of topical hydroquinone-containing cream (38–40). This color change of the nail was considered to be due to the oxidation products of hydroquinone. Hydroquinone readily oxidized to quinone and subsequently to hydroxyquinone, which is unstable and polymerizes to a brown compound.

CONCLUSIONS

In this report we have provided evidence that currently used skin-depigmenting agents have drawbacks in either their efficacy or their safety. Deoxyarbutin is demonstrated to be less cytotoxic than the standard skin-lightening product in the market (i.e., HQ). The cytotoxicity dA exhibited is not associated with dramatic degenerative changes in the morphology of keratinocytes as compared with HQ. Our overall goal is to provide the market with an effective skin depigmenting agent due to the fact that available depigmenting agents in the market are far from satisfactory. We have clearly demonstrated that dA has the potential to be a safe and effective depigmenting agent. It also offers the potential to be an effective alternative to hydroquinone, the skin-depigmenting standard in the market with known safety drawbacks.

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